Universidade de Aveiro Departamento de Biologia
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Luísa Maria Pires dos Santos

Influência hidrológica e fotoquímica na dinâmica bacteriana estuarina

Hydrological and photochemical influence on dynamics of estuarine bacteria

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutora Maria Adelaide Pinho de Almeida, Professora auxiliar do Departamento de Biologia da Universidade de Aveiro e, co-orientação científica da Doutora Maria Ângela Sousa Dias Alves Cunha, Professora auxiliar do Departamento de Biologia da Universidade de Aveiro e do Doutor João Miguel Sequeira Silva Dias, Professor auxiliar do Departamento de Física da Universidade de Aveiro.

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Dedico este trabalho ao meu pai (em memória)

o júri

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palavras-chave dinâmica bacteriana, hidrologia, água doce, microcamada superficial, fotoquímica, matéria orgânica dissolvida, estuário, Ria de Aveiro

resumo

Os estuários são ecossistemas complexos, onde os processos físicos, químicos e biológicos estão intimamente ligados. A dinâmica bacteriana num estuário reflete a interação e a elevada variação temporal e espacial desses processos.

Este trabalho teve como objetivo elucidar as interações entre os processos físicos, fotoquímicos e microbiológicos no sistema estuarino da Ria de Aveiro (Portugal). Para tal, foi realizada uma abordagem inicial no campo, durante a qual as comunidades bacterianas na coluna de água foram caracterizadas em termos de abundância e atividade ao longo de 2 anos. O estudo foi realizado em dois locais distintos, escolhidos por tipificarem as características marinhas e salobras do estuário. Estes locais possuem diferentes hidrodinâmicas, influências fluviais e, quantidade e composição de matéria orgânica. Numa perspectiva mecanicista, foram realizadas simulações laboratoriais no sentido de elucidar a resposta das bactérias à matéria orgânica foto-transformada.

As comunidades bacterianas no estuário adaptam-se a diferentes regimes de água doce, desenvolvendo padrões de abundância e atividade distintos nas zonas marinha e salobra. Os elevados caudais dos rios induzem estratificação vertical na zona marinha, promovendo o fluxo de fitoplâncton do mar para o estuário, do bacterioplâncton do estuário para o mar, e estimulam a importação de bactérias aderentes a partículas na zona salobra. O transporte advectivo e os processos de ressuspensão contribuem para aumentar 3 vezes o número de bactérias aderentes a partículas durante os períodos de intensas descargas fluviais. Adicionalmente, a atividade bacteriana no estuário é controlada pela concentração de azoto inerente à variações de água doce. O fornecimento de azoto em associação com a fonte dos substratos bacterianos induzem alterações significativas na produtividade.

O padrão de variação vertical de comunidades bacterianas foi distinto nas duas zonas do estuário. Na zona marinha, as bactérias na microcamada superficial (SML) apresentaram taxas de hidrólise mais elevadas, mas menores taxas de incorporação de monómeros e produção de biomassa que na água subjacente (UW), enquanto na zona salobra, as taxas de hidrólise e incorporação foram similares nos dois compartimentos, mas a produtividade foi significativamente mais elevada na SML. Apesar da abundância bacteriana ter sido semelhante na SML e UW, a fração de células aderentes a partículas foi significativamente maior na SML (2-3 vezes), em ambas as zonas do estuário. A integração dos resultados microbiológicos com as variáveis ambientais e hidrológicos mostraram que fortes correntes na zona marinha promovem a mistura vertical, inibindo o estabelecimento de uma comunidade bacteriana na SML distinta da UW. Em contraste, na zona de água salobra, a menor velocidades das correntes fornece as condições adequadas ao aumento da atividade bacteriana na SML. Características específicas do local, tais como a hidrodinâmica e as fontes e composição da matéria orgânica, conduzem também a diferentes graus de enriquecimento superficial de matéria orgânica e inorgânica, influenciando a sua transformação. Em geral, o ambiente da SML estuarina favorece a hidrólise de polímeros, mas inibe a utilização de monómeros, comparativamente com água subjacente. No entanto, as diferenças entre as duas comunidades tendem a atenuar-se com o aumento da atividade heterotrófica na zona salobra.

A matéria orgânica dissolvida cromófora (CDOM) das duas zonas do estuário possui diferentes características espectrais, com maior aromaticidade e peso molecular médio (HMW) na zona de água salobra, em comparação com a zona marinha. Nesta zona, a abundância bacteriana correlacionou-se com a_{350} e a_{254}, sugerindo uma contribuição indireta das bactéria para HMW CDOM. A irradiação do DOM resultou numa diminuição dos valores de a_{254} e a_{350}, e, em um aumento do declive $S_{275-295}$ e dos rácios E_2:E_3 (a_{250}/a_{365}) e S_R. No entanto, a extensão de transformações foto-induzidas e as respostas microbianas são dependentes das características iniciais CDOM, inferidas a partir das suas propriedades ópticas.

resumo (cont.)

A dinâmica estuarina influencia claramente as atividades heterotróficas e a distribuição dos microorganismos na coluna de água. A entrada de água doce influencia a dinâmica e os principais reguladores das comunidades bacterianas no estuário. Os processos fotoquímicos e microbianos produzem alterações nas propriedades ópticas da CDOM e a combinação desses processos determina o resultado global e o destino da CDOM nos sistemas estuarinos com influência na produtividade nas áreas costeiras adjacente.

keywords

bacterial dynamics, hydrology, freshwater, surface micolayer, photochemistry, dissolved organic matter, estuary, Ria de Aveiro

abstract

Estuaries are complex ecosystems where physical, chemical and biological processes are tightly connected. Dynamics of estuarine bacteria reflect the interaction and high spatial and temporal variation of these processes.

This work aimed to elucidate the interactions between physical, photochemical and microbiological processes in the estuarine system Ria de Aveiro (Portugal). For that, an initial field-approach was developed during which bacterial communities in the water column were characterised in terms of abundance and activity during a 2-year survey. The study was conducted in two sites chosen to typify the characteristics of the marine and brackish zones of the estuary, with distinct hydrodynamics, freshwater influences and prevailing sources, amounts and composition of organic matter. For a mechanistic perspective, laboratory simulations were performed in order to elucidate the response of estuarine bacteria to photo-transformed dissolved organic matter (DOM).

Estuarine bacterial communities adapt to different freshwater regimes by developing distinct patterns of abundance and activity at marine and brackish water zones. A circulation pattern induced by high river inflow produced vertical stratification at the marine zone, promoting a landward flux of phytoplankton and seaward flux of bacterioplankton, stimulating the import of particle-attached bacteria to the brackish water zone. Advective transport and resuspension processes contributed to a 3-times increase of the abundance of particle-attached bacteria during intense freshwater inputs. Additionally, bacterial activity in the estuary was controlled by nitrogen concentration, responding to different freshwater inputs. Nitrogen supply in association with shifting sources of organic substrates, induce significant changes in bacterial production.

The vertical pattern of variation of bacterial communities was distinct between the two estuarine zones. At the marine zone, bacteria in the surface microlayer (SML) exhibited higher rates of hydrolysis, but lower rates of monomer incorporation and biomass production than in underlying water (UW), whereas at the brackish water zone, the rates of hydrolysis and incorporation were similar in the two compartments, but biomass productivity was significantly enhanced at the SML. Although the total bacterial abundance was similar in the SML and UW, the fraction of cells attached to particles was significantly higher at the SML (two to three times) at both estuarine zones. The integration of microbiological results with environmental and hydrological variables shows that strong currents in the marine zone promote the vertical mixing, inhibiting the establishment of an SML bacterial community distinct from that of UW. In contrast, in the brackish water zone, lower current velocities provide conditions for enhancing the bacterial activity in the enriched SML. Site-specific characteristics, such as hydrodynamics and sources of organic matter composition, also lead to different degrees of surface organic and inorganic matter enrichments, influencing the organic matter transformation. In general, estuarine SML environment favors polymer hydrolysis, but inhibits monomer utilisation, in comparison with the sub-surface water layers. However, the differences between the two communities tend to attenuate as heterotrophic activities increase in the brackish-water area.

The colored dissolved organic matter (CDOM) of the two estuarine zones showed different spectral characteristics, with higher aromaticity and average molecular weight (HMW) at the brackish water zone, in comparison with the marine zone. At the marine zone, the abundance of bacteria correlated with a_{350} and a_{254}, suggesting an indirect microbial contribution to the HMW CDOM pool. The irradiation of DOM resulted in a decrease of the values of a_{254} and $a_{350,}$ and, in an increase of the slope $S_{275\text{-}295}$ and of the ratios E_2:E_3 (a_{250}/a_{365}) and S_R. However, the extent of photo-induced transformations and microbial responses was dependent on the initial characteristics of CDOM as inferred from the optical properties.

abstract (cont.)

Estuarine dynamics clearly influenced the distribution and heterotrophic activities of microorganisms along the water column. The dynamics and the main factors of regulation of bacterial communities in the estuary are impacted by freshwater inputs. Photochemical and microbial processes yielded changes in the optical properties of CDOM and the overall result of these combined processes may determine the fate of CDOM in the estuarine system and have influence on local productivity and in the adjacent coastal areas.

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ABBREVIATION	TRANSLATION
a_λ	- the absorption coefficient at λ
BBP	- bacterial biomass production
CDOM	- colored dissolved organic matter
DIC	- dissolved inorganic carbon
DIN	- dissolved inorganic nitrogen
DOC	- dissolved organic carbon
DOM	- dissolved organic matter
HMW DOM	- high molecular weight DOM
Leu-AMPase	- aminopeptidase
LMW DOM	- low molecular weight DOM
OM	- organic matter
PAB	- particle-attached bacteria
POC	- particulate organic carbon
POM	- particulate organic matter
SML	- surface microlayer
SPM	- suspended particulate matter
SRP	- soluble reactive phosphorus
ß-GlCase	- ß-glucosidase
$SUVA_{254}$	- specific ultra-violet absorbance at 254 nm
TBN	- total bacterial number
UV-A	- UVR in the wavelength range 315-400 nm
UV-B	- UVR in the wavelength range 280-315 nm
UVR	- ultraviolet radiation
UW	- underlying water
λ	- wavelength

1. INTRODUCTION

Hydrodynamic, geochemical and biological processes in estuarine systems are tightly coupled and modulated by a wide variety of forcing mechanisms such as light, temperature, wind stress, waves, tides, freshwater discharge, as well as continental and oceanic nutrient inputs (Arndt *et al.*, 2011).

Estuarine hydrodynamics considers the circulation and mixing within an estuary in response to freshwater inputs via river discharge and injection of saline waters through tidal flows. In addition, heat inputs during high insolation periods enhance vertical stratification (Statham, 2012). Estuarine hydrodynamics have influence on nutrient availability, through transport processes, on light penetration, on deposition/erosion of particulate matter and will ultimately determine how long will a water mass will persist in a particular estuarine section (i.e. the residence time (Deleersnijder *et al.*, 2001; Braunschweig *et al.*, 2003).

Biogeochemical processes within estuaries range from interactions with biota (fixed macro and water column phytoplankton, plus bacteria) and exchanges with suspended particles and sediment systems, to photochemistry and exchanges of gases across the atmosphere–water–sediment boundaries (Statham, 2012).

As key players in aquatic biogeochemical cycles (Cho & Azam, 1988), microbes are critical in controlling the function and structure of estuarine ecosystems through activities that include nutrient cycling, organic matter decomposition and conversion of indigestible detrital materials more accessible to large consumers (Blum & Mills, 2012). Photochemical processes act in concern with microbial processes in organic matter alteration and removing pathways (Wiegner & Seitzinger, 2001; Amado *et al.*, 2006; Vahatalo & Wetzel, 2008) and assume a particular importance in estuarine systems, due to the large inputs of terrestrial organic carbon (Hedges *et al.*, 1997) that are processed during transport to the ocean (Wiegner & Seitzinger, 2001).

Therefore, in order to understand the dynamics of estuarine bacteria and determine their influence on biogeochemical cycles in these ecosystems, it is essential to understand the mechanisms that control their spatial and temporal variability, as well as the influence of physical and chemical processes on these dynamics.

1.1. DYNAMICS OF ESTUARINE BACTERIA

1.1.1. ABUNDANCE

The study of patterns in the distribution of organisms in space, and their fluctuations in time are essential to understand the ecological dynamics of populations (Ducklow *et al.*, 1999). In an estuarine system, the spatial distribution of bacteria is the result of the interactions between several dynamic processes such as growth, mortality, predation, and physical dispersion (i.e. advection and diffusion) (Painchaud *et al.*, 1996). Bacterial abundance in estuaries varies widely, ranging from 0.2 to 15.3 $x10^9$ cells L^{-1} (Hollibaugh & Wong, 1999; Revilla *et al.*, 2000; Murrell, 2003; Bordalo & Vieira, 2005; Barrera-Alba *et al.*, 2008; Barrera-Alba *et al.*, 2009; Lapoussière *et al.*, 2011).

A typical longitudinal pattern of bacterial abundance in an estuary fits in a curvilinear pattern with a peak in the mesohaline zone or in a conservative mixing behaviour of decrease with salinity gradient. A mid-estuarine maximum of abundance has been observed in the Delaware Estuary (Kirchman & Hoch, 1988), Essex river estuary (Wright & Coffin, 1983), Chesapeake Bay (Ducklow *et al.*, 1999) and Ria de Aveiro (Cunha *et al.*, 2000). Decreasing bacterial abundances from the upper to lower estuary were observed in the Schelde (Goosen *et al.*, 1997), Upper St. Lawrence (Painchaud & Therriault, 1989; Painchaud *et al.*, 1995b; Painchaud *et al.*, 1996) and Urdaibai (Revilla *et al.*, 2000) estuaries. However, other uncharacteristic profiles of variation were identified such as in the York River, a sub-estuary of Chesapeake Bay, where bacterial abundance increases from the freshwater to the mouth (Ducklow *et al.*, 1999).

Despite of the low vertical variation in estuaries (Almeida *et al.*, 2001a; Barrera-Alba *et al.*, 2008), the abundance of bacteria could be significantly higher (Santos *et al.*, 2009) at the uppermost centimeter of the water column, the surface microlayer (SML) (Liss & Duce, 1997). However, similar values in SML and respective underlaying water (UW) were also observed in estuaries (Bell & Albright, 1982).

1.1.2. ACTIVITY

Characteristic gradients of organic carbon and inorganic nutrients in estuaries support high rates of primary (Boyer *et al.*, 1993; Sorokin & Sorokin, 1996; Gaulke *et al.*, 2010) and secondary production (Goosen *et al.*, 1999; Almeida *et al.*, 2001a; Almeida *et al.*, 2002a; Ducklow, 2002).

Bacterial biomass production in estuaries showed a wide range of variation (0.08 to 63.8 µg C L^{-1} h^{-1}), displaying patterns of longitudinal and vertical variation (Hollibaugh & Wong, 1999; Revilla *et al.*, 2000; Murrell, 2003; Almeida *et al.*, 2007; Barrera-Alba *et al.*, 2008; Barrera-Alba *et*

al., 2009).

Estuarine typical longitudinal patterns of activity presented a linear or a curvilinear relation with salinity, similarly to bacterial abundance. Peaks of bacterial production at intermediate salinities were observed in mid-Bay region of Chesapeake Bay (Ducklow *et al.*, 1999), in Mississippi River plume (Chin-Leo & Benner, 1992), in Neuse River – Pamlico Sound estuary (Peierls & Paerl, 2011) and Ria de Aveiro (Almeida *et al.*, 2002a). The location and amplitude of the peak varies seasonally (Chin-Leo & Benner, 1992) and inter-annually, it is clear even in annually averaged data and corresponds to the axial distribution of chlorophyll (Ducklow *et al.*, 1999; Peierls & Paerl, 2011). A linear decrease of heterotrophic activity in the seaward direction was observed in Schelde (Goosen *et al.*, 1997), York River (Ducklow *et al.*, 1999), Upper St. Lawrence estuaries (Painchaud & Therriault, 1989) and Urdaibai (Revilla *et al.*, 2000) estuaries.

Bacterial production showed a typical profile of longitudinal variation with lower rates in the lower and higher in the upper estuary, increasing 10 times from the middle to upper compared with the lower estuary (Goosen *et al.*, 1997).

Estuarine vertical profiles of varition of bacterial activity showed usually maximum values in the surface and middle-depth of the water column (Almeida *et al.*, 2001a; Barrera-Alba *et al.*, 2008; Peierls & Paerl, 2011). Compared with the underlaying water column, the heterotrophic activity of bacteria in the estuarine SML environment could be enhanced (Santos *et al.*, 2009) or reduced (Bell & Albright, 1982).

1.2. FACTORS OF REGULATION OF ESTUARINE BACTERIA

The primary factors regulating estuarine bacterial communities activity and abundance are highly variable among systems and include salinity (del Giorgio & Bouvier, 2002; Langenheder *et al.*, 2003; Apple *et al.*, 2008), temperature (Hoch & Kirchman, 1993; Shiah & Ducklow, 1994; Apple *et al.*, 2006; Apple *et al.*, 2008), organic matter source and quality (Raymond & Bauer, 2000; Apple & del Giorgio, 2007), anthropogenic nutrient inputs (Revilla *et al.*, 2000) and light availability (Harding Jr *et al.*, 1986).

1.2.1. PHYSICAL AND CHEMICAL

Physical and chemical factors are important selective forces for bacterial communities. Temperature, in particular, should be viewed as an ever-present, interactive factor, because it affects all chemical and biochemical processes (Pomeroy & Wiebe, 2001). The effect of

temperature on bacterial carbon metabolism in estuaries has been the subject of numerous studies (Hoch & Kirchman, 1993; Sampou & Kemp, 1994; Shiah & Ducklow, 1994; Raymond & Bauer, 2000; Apple *et al.*, 2006). Generally, temperature dependence of bacterial growth and production is stronger at lower temperatures and is often modulated by other environmental conditions, namely the availability of inorganic nutrients and, the quality and quantity of organic matter substrates (Apple *et al.*, 2006). In the Delaware Estuary, the dependence of growth rate on temperature was particularly evident during cold months (< 12 ºC), but once the water warmed above 12°C, growth rate became independent of temperature (Hoch & Kirchman, 1993). However, in temperate latitudes, bacterial metabolism in winter may also be limited by lower rates of dissolved and particulate organic carbon by phytoplankton as a result of lower light intensity and deep mixing, and these limiting factors may interact synergistically with lower temperature (Pomeroy & Wiebe, 2001).

The effect of temperature also depends on the metabolic activity parameter determined. In the temperate estuarine system Monie Bay (Chesapeake Bay, USA), temperature was the dominant factor regulating seasonality of bacterial respiration and carbon consumption, whereas biomass production and growth efficiency was influenced by water temperature and the quality of organic pool, with variation in the relative importance of each of these factors throughout the year (Apple *et al.*, 2006). Therefore, temperature is always a factor in microbial growth, respiratory rate, and organic carbon assimilation, but it is not always the only factor or even a dominant one (Pomeroy & Wiebe, 2001).

In estuaries, the marine and freshwater mixing produces steep gradients of salinity that may range from 0 psu to near marine salinities over short distances. Along this gradient, bacterial communities experience significant phylogenetic (Bouvier & del Giorgio, 2002; Troussellier *et al.*, 2002; Henriques *et al.*, 2006) and metabolic changes (Schultz & Ducklow, 2000; Almeida *et al.*, 2001b; Cunha *et al.*, 2001), induced by salinity as a possible physiological stress factor (del Giorgio & Bouvier, 2002). Even moderate changes of salinity could have pronounced impacts on both composition and functional performance of bacterial communities (Langenheder *et al.*, 2003). For example, changes in salinity produced considerable physiological stress on riverine bacteria, affecting their performance and functioning (Painchaud *et al.*, 1995a; del Giorgio & Bouvier, 2002). Salinity change may act as a filter on bacterial communities, resulting in the 'loss' of some species but also in the 'activation' of others (Langenheder *et al.*, 2003). del Giorgio and Bouvier (2002) showed that salinity affects selectively certain phylogenetic groups, which respond to changing salinity with a marked decrease in cell-specific activity and growth efficiency. However, a change of functional performance could occur without significant community composition alterations. Changes in ionic strength can affect the activity of extracellular enzymes and the structure of substrates molecules (Langenheder *et al.*, 2003), affecting the ability for bacterial DOC

utilisation and consequently their growth and biomass production. The effect of salinity on estuarine bacterial communities could be also indirect as result of physicochemical changes in DOC composition, such as molecular aggregation of riverine DOC during estuarine transport (Fox, 1983; Benner & Opsahl, 2001).

1.2.2. ORGANIC SUBSTRATES (MULTIPLICITY OF SOURCES, BIOAVAILABILITY)

Organic matter in estuaries derives from multiple natural and anthropogenic allochthonous and autochthonous sources that originate across a freshwater to seawater continuous (Bianchi, 2006). Allochthonous organic matter is transported from the surrounding landscape to the water body, and is derived from and influenced by the geology, land use and hydrology of its origin (Aitkenhead-Peterson *et al.*, 2003; Hudson *et al.*, 2007). Autochthonous organic matter is created *in situ* and in estuaries, the major sources are primary production by phytoplankton, microphytobenthos and higher plants, chemoautotrophic production by nitrifiers and secondary production by bacteria and zooplankton (Middelburg & Herman, 2007). Secondary production may provide an independent source of organic matter, or a recycling mechanism of allochthonous organic matter (Hudson *et al.*, 2007). Phytoplankton mechanisms of dissolved organic matter (DOM) production include (1) active excretion of photosynthetic products, (2) solubilization of senescent phytoplankton cells and sinking detritus, (3) rupture of cells due to grazing and (4) cell lysis by pathogens (Amon, 2002). The relative proportion of each source depends upon the location (Mannino & Harvey, 1999; McCallister *et al.*, 2006b), season (McCallister *et al.*, 2004; McCallister *et al.*, 2006b) and hydrological characteristics (McKenna, 2004) of the systems. Stable isotope analysis, and fatty acids and sterol distributions of organic matter in the York river estuary showed that organic matter in the mid- and high salinity regions displayed a more pronounced algal (diatom) signatures while the riverine end-member reflected the importance of both higher plants and algal sources other than diatoms (McCallister *et al.*, 2006b), evidencing the spatial variability and the multiplicity of organic sources in the estuarine systems. Human activity is also a source of DOM, which can enter the aquatic system through direct point discharges, diffuse leaching and aerial dispersal (Hudson *et al.*, 2007).

Organic matter bioavailability is a result from a complexity of intrinsic factors, such as the chemical characteristics of the DOM itself, which include the molecular weigh distribution, the nutrient contents, and the relative contribution of a broad class of compounds and are determined by the source and the diagenetic state of the matter (Amon & Benner, 1996; Amon *et al.*, 2001). Hopkinson and co-workers (1998) observed that between 67% to 75% of the variation in bacterial growth in estuaries could be explained by differences in organic matter composition. The source and quality of organic matter also play an important role in regulating the magnitude of carbon

metabolism in a carbon-rich estuary (Apple & del Giorgio, 2007). Estuarine dissolved organic carbon (DOC) pools relatively enriched with proteinaceous compounds are of higher quality than pools dominated by dissolved carbohydrates derived from complex plant polysaccharides or amino sugars (Hopkinson *et al.*, 1998). Contrarily to the DOM derived from vascular plants, considered of refractory nature (Moran & Hodson, 1994), DOM produced by algae is very labile and subject to high turnover rates by heterotrophic bacteria. Despite the fact that geochemical signature of algal-derived organic matter (OM) could be quantitatively insignificant, this fraction represents nevertheless a principal source of bioreactive OM to heterotrophic bacteria in estuarine waters (McCallister *et al.*, 2006a). However, autochthonous DOM is sometimes insufficient to support bacterial growth, particularly during the low primary productivity season, when bacterioplankton biomass production rely on non-phytoplankton or allochthonous organic matter supplies in estuarine systems (Almeida *et al.*, 2002a; Barrera-Alba *et al.*, 2009). The availability of allochthonous DOC under estuarine conditions varies with season and flow regime, with highest utilisation by bacteria coincided with spring flood, when about half of annual riverine DOC discharges occur (Wikner *et al.*, 1999). Changes in ionic strength of the milieu, similar of occurring during the fresh and seawater mixing in estuaries could "active" sites for degradation, increasing the the accessibility and therefore the availability of allochthonous DOC (Boyd & Osburn, 2004). Additionally, photochemical processes could enhance bacterial utilisation of biorefractory DOM (Benner & Ziegler, 1999).

1.2.3. Photochemical Processes

Photochemical processes induce changes in natural DOM, influencing carbon cycling in estuarine environments by several ways. Light-induced photochemical reactions result in the reduction of DOC average molecular weight (Lou & Xie, 2006), changes in water optical properties (Osburn *et al.*, 2001; Yunlin *et al.*, 2009), and in the production of reactive oxygen species (Aguer *et al.*, 1999) and carbon photoproducts (Johannessen & Miller, 2001; Zhang *et al.*, 2006; White *et al.*, 2010), many serving as biological substrates (Kieber & Mopper, 1987; Miller & Moran, 1997; Zhou & Mopper, 1997; de Bruyn *et al.*, 2011). Several studies showed that solar irradiance may cleave recalcitrant high molecular weight (HMW) DOM into smaller fractions, and thereby facilitate bacterial utilisation with the result of an increased bacterial production (Bushaw *et al.*, 1996; Bano *et al.*, 1998; McCallister *et al.*, 2005). A variety of low molecular weight (LMW) carboxylic acids (citric, pyruvic, and levulinic, oxalic, malonic, formic, and acetic acid among others) has been identified as photoproducts of DOM (Wetzel *et al.*, 1995; Bertilsson & Tranvik, 1998, 2000). Moreover, photochemical processes change the bioavailability of DOM. HMW compounds and humic substances in DOM pool may be modified by sunlight exposure (Miller & Moran, 1997), increasing their accessibility to microbial enzymes.

The irradiation of DOM, particularly by UV-B and UV-A, also results in a progressive loss of colour (photobleaching) induced by absorption of solar radiation or promoted by photoproduced oxidants (Zagarese *et al.*, 2001). Photobleaching processes lead to the increasing of water column transparency, exposing aquatic organisms to ultraviolet radiation (UVR) harmful consequences (Zepp *et al.*, 2007). With higher solar radiation exposition and CDOM concentrations compared to the rest of water column, the SML compartment has higher photoproduction rates and concentrations of LMW compounds compared with UW (Zhou & Mopper, 1997).

1.2.4. HYDROLOGY

In order to understand the activity and distribution of organisms in estuaries it is essential to understand the processes that influence water movement (e.g. evaporation, precipitation, riverine discharge, submarine water discharge, wetland hydrology and tidal exchange) as well as other hydrodynamics aspects of coastal systems, including circulation patterns, stratification, mixing and flushing (Snedden *et al.*, 2012). The study of estuarine hydrology is challenging due to complex circulation and mixing patterns, produced by the freshwater runoff interaction with tidal saltwater exchange and variable winds. Moreover, these patterns are shaped by climatic forcing features (i.e., temperature, rainfall, winds, etc.) that vary over multiple time and space scales and strongly influence the chemical and biological characteristics and responses of these ecosystems to environmental changes and perturbations (Paerl *et al.*, 2010).

Freshwater flow is the predominant source of seasonal and inter-annual variability in estuaries (Skreslet, 1986), influencing the physics, geology, chemistry, and biology of estuaries through a variety of pathways (Skreslet, 1986; Sklar & Browder, 1998). Changes in freshwater flow impact the inundation of the flood plains, advective transport of materials and organisms, dilution or mobilisation of contaminants, compression of the estuarine salinity field and density gradient, stratification, and residence time for water (Kimmerer, 2002) and ultimately, estuarine microbial processes. The seasonal reduction of freshwater water inflow caused a sharp temporal decline in particle-associated bacteria in S. Francisco estuary, due to the combined effects of loss of nutritive value of particles, a decrease in advection of free-living bacteria, and cumulative benthic grazing pressure (Murrell *et al.*, 1999). An increase of freshwater inputs to the Ria de Aveiro, during a particularly rainy year, caused alterations in the seasonal and spatial patterns of bacterial activity in the estuary, influenced by the high loads of inorganic nutrients and allochthonous organic substrates and, by changes in the salinity (Almeida *et al.*, 2007). Moreover, freshwater discharge affects the residence time available for the different planktonic organisms grow, influencing succession between marine, estuarine and freshwater phytoplankton (Chan & Hamilton, 2001; Roelke *et al.*, 2013) and bacterioplankton (Crump *et al.*, 2004) taxa.

Besides of freshwater inflow, estuarine short-term and more local physical processes such as sediment resuspension driven by wind, may also influence the distribution and variability of estuarine bacteria. In St. Lawrence Estuary, large-scale processes, such as freshwater outflow and residual circulation, largely control free bacteria, whereas short-term and more local processes (e.g., sediment resuspension caused by wind) may also be important in the control of attached bacteria (Painchaud *et al.*, 1995b).

1.3. THE CASE OF RIA DE AVEIRO

With 10 km with and 45 km length, the Ria de Aveiro (40" 38' N, 8" 44' W; Figure 1.1) is the largest coastal lagoon in Portugal and the most dynamic in terms of physical and biogeochemical processes (Dias *et al.*, 2003; Vaz *et al.*, 2005). Covering an area of 83 km^2 and 66 km^2 at high and low tide, respectively, it has a very irregular and complex geometry, characterized by narrow channels and by the existence of significant intertidal zones, namely mud flats and salt marshes (Dias *et al.*, 1999). It is connected with the Atlantic through an artificial channel and exchanges the most part of its water with the ocean by tidal input across this narrow entrance, 1.3 km long, 350 m wide and 20 m deep. The average depth of the lagoon is about 1 m, except in navigation channels where dredging operations are frequently carried out (Dias *et al.*, 1999).

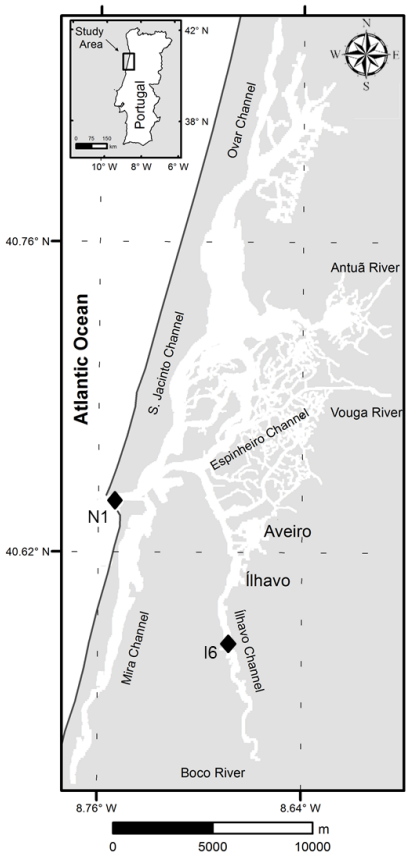

Figure 1.1. The estuarine system Ria de Aveiro with indication of sampling stations. Station N1 in Canal de Navegação represents the marine zone, and station I6, in Canal de Ílhavo, represents the brackish water zone.

The lagoon has four main branches radiating from the sea entrance: Mira, S. Jacinto, Ílhavo and Espinheiro channels. The Mira channel is an elongated shallow arm, with 20 km length, which receives continuous freshwater input at its far end from a small system of ponds and rivers. With approximately 29 Km length, the S. Jacinto channel is the most important channel in terms of average width and length. The Ílhavo channel is the narrower and shorter of the main channels, with a length of 15 km. The Espinheiro is a short channel of about 17 km in length and has a complex network of small dead arms (Dias *et al.*, 1999).

Freshwater to lagoon is supplied mainly by rivers Vouga, Antuã, Caster, Gonde and Boco, which discharge an average water input of 1.8 Mm^3 during a tidal cycle (Dias *et al.*, 2003). Of these rivers, the major contributor is Vouga River which discharges more than 66% of the incoming freshwater (Dias *et al.*, 1999) and is connected to the Atlantic Ocean by the Espinheiro channel.

1.3.1. HYDROLOGY

The main forcing action driven water circulation in Ria de Aveiro is the astronomical tide (Dias *et al.*, 1999). Tides are semidiurnal, with a mean tidal range of about 2.0 m. At the mouth of the lagoon, the minimum tidal range is 0.6 m (neap tides), and the maximum tidal range is 3.2 m (spring tides), corresponding to a maximum and a minimum water level of 3.5 and 0.3 m, respectively (Dias *et al.*, 1999). According with this values, Ria de Aveiro was classified as a mesotidal lagoon (Dias *et al.*, 1999). The tidal prism at the mouth in a spring tide with a tidal range of 2.48 m is about 70 x 10^6 m^3 and the relative contribution of each of the main channels to its value at the mouth is about 38 % for the S. Jacinto channel, 26 % for the Espinheiro channel, 10 % for the Mira channel and 8 % for the Ílhavo channel (Dias *et al.*, 1999). Tides propagate from the mouth of Ria de Aveiro and are present in the entire lagoon and generate strong currents in deep and narrow channels, but not in the intertidal area. The highest values are found at the beginning of Espinheiro and S. Jacinto channels, and specially in the entrance channel, where the tidal current velocity can be higher than 1 m/s (Dias *et al.*, 2000). The strength and magnitude of tidal currents in the estuary depend essentially on the cross sectional area and depth of the channels (Dias *et al.*, 1999).

Despite of the tidal predominance, freshwater inputs from the main rivers during flooding situations have also important consequences in the establishment of the water circulation patterns in the lagoon (Dias *et al.*, 2003). The total mean river discharge during a tidal cycle into the lagoon is about $1.8x10^6$ m^3 (Dias *et al.*, 1999) and although the rivers have a small contribution in terms of water input, when compared to the tidal prism, they may have a long-term influence in the residual transport (Dias *et al.*, 2003).

The residence time in Ria de Aveiro clearly reveals the influence of the tidal currents. Residence times less than 2 days are almost entirely confined to the central area of the lagoon (beginning of Mira and Ílhavo channels and almost half of S. Jacinto and Espinheiro channels). The lowest values are found close to the mouth of Ria de Aveiro, where the tidal currents are very strong. Therefore, the water in these areas is rapidly renewed. The residence time pattern determined for Mira and Ílhavo channel is similar, with the values strongly increasing after the first kilometers towards the end of the channels; where they are higher than 14 days. The gradient

observed for S.Jacinto channel is much more smoothed, with intermediate values of the order of 1 week found in a large zone close to Torreira and where the water renewal is small (Dias *et al.*, 2001).

1.3.2. CONCENTRATION OF ORGANIC AND INORGANIC NUTRIENTS

Typically referred as a 'mesotrophic' estuarine system, the Ria de Aveiro has a trophic status in the inner higher than in the outer sections (Lopes *et al.*, 2007b).

Based on chlorophyll a concentration and, winter season average concentrations of phosphate and dissolved inorganic nitrogen (DIN), Lopes and co-workers (2007b) determined that the inner areas of the estuary fall into the "eutrophic" status and the outer boundary into 'mesotrophic', applying the trophic status criteria proposed by Wasmund and co-workers (2001).

1.3.2.1. SUSPENDED PARTICULATE MATTER

The concentration of suspended particulate matter (SPM) in Ria de Aveiro ranged from 7 to 241 mg L^{-1} (Cunha *et al.*, 2000; Almeida *et al.*, 2001a; Almeida *et al.*, 2002b; Cunha *et al.*, 2003b; Cunha *et al.*, 2003a; Lopes *et al.*, 2008), with no clear temporal and spatial pattern of variation. In the Mira and Espinheiro channels was observed, nonetheless, a decrease tendency towards the landward end (Almeida *et al.*, 2007). The maximal values were generally found at the bottom of the water column (Cunha *et al.*, 2003b). The concentration of SPM in the estuary fluctuated strongly during the tidal cycles and a clear pattern could not be identified (Cunha *et al.*, 2003a), however, at high tide, a considerable decrease in the amount of SPM was observed in the inner- compared to in the outer-estuary. In the outer- and mid-estuary, the SPM content at low was up to 16% greater than at high tide. In the inner estuary the corresponding increase was greater (37 or 44%), denoting a richer particle content of the inflowing river (Cunha *et al.*, 2000). There was no clear pattern of SPM variation among spring tides or neap tides (Almeida *et al.*, 2002b; Lopes *et al.*, 2008).

1.3.2.2. PARTICULATE ORGANIC CARBON

The concentration of particulate organic carbon (POC) in Ria de Aveiro ranged from 0.3 to 15.5 mg L^{-1} (Cunha *et al.*, 2000; Almeida *et al.*, 2001a; Almeida *et al.*, 2002b; Cunha *et al.*, 2003b; Cunha *et al.*, 2003a; Lopes *et al.*, 2008) and represent 5 and 20 % of the SPM (Cunha *et al.*, 2000; Almeida *et al.*, 2002b), averaging 10% at mouth of the estuary (station N1) and 9.7% at inner

estuary (station I6) (Almeida *et al.*, 2002b). At station N1, the concentration of POC was generally higher in deep water when at low tide, but at station I6, the values were similar down the water column (Almeida *et al.*, 2002b). POC maximal concentrations at stations N1 and I6 were observed two hours before low tide and the minimal at high tide (Almeida *et al.*, 2002b). At station N1, POC concentration was similar between neap and spring tides (Almeida *et al.*, 2002b; Lopes *et al.*, 2008), but at station I6 the values were, on average, 30% higher at spring tide (Almeida *et al.*, 2002b).

1.3.2.3. DISSOLVED ORGANIC CARBON

The concentration of dissolved organic carbon (DOC) in the estuary Ria de Aveiro ranged from 1 to 77.9 mg L^{-1} (Almeida *et al.*, 2002b; Almeida *et al.*, 2007; Lopes *et al.*, 2008), without any clear temporal or spatial pattern of variation (Almeida *et al.*, 2007; Lopes *et al.*, 2008). Compared with the station N1, the concentration of DOC at the station I6, was 2.5 times higher (Almeida *et al.*, 2002b). In the estuary, DOC concentration is similar along the water column. However, in the inner section of the estuary, DOC concentration is 2.3 times, on average, higher at the channel margin compared with mid-channel values (Almeida *et al.*, 2002b).

1.3.2.4. INORGANIC NUTRIENTS

Dissolved inorganic nitrogen (DIN) in Ria de Aveiro show a strong spatial and seasonal variation and 67 % is constituted by nitrate (Lopes *et al.*, 2007a). DIN concentration in the estuary correlated negatively with salinity establishing a freshwater source (Cunha & Almeida, 2009) and suggesting a conservative mixing between DIN-rich estuarine waters and DIN-poor coastal waters and the absence of significant sources or sinks of DIN in near-mouth region (Lopes *et al.*, 2007a). The concentration of nitrate plus nitrite varied between 0.9 and 204.6 µM with maximum values occurring from late autumn to winter and minimum in late spring (Almeida *et al.*, 2002a; Almeida *et al.*, 2007; Lopes *et al.*, 2007a; Cunha & Almeida, 2009). Nitrate concentrations depended on tidal conditions, with the highest values recorded at low tide (ratio low/high tide of 2.3) (Almeida *et al.*, 2001a; Almeida *et al.*, 2002a; Lopes *et al.*, 2007a). Ammonium varied between 2.0 and 142.0 µM and the highest values occurred in October (Cunha & Almeida, 2009). It was estimated that the estuary receives an annual influx of total N of about 6118 t y^{-1} from its influent rivers, but in low summer flows, the flux decreases to about 10% of the annual average (Silva *et al.*, 2002).

Compared with DIN, the concentration of phosphate in the estuary Ria de Aveiro is not so clearly seasonally and spatially patterned, ranging from 0.3 to 137 µM (Almeida *et al.*, 2001a; Almeida *et al.*, 2002a; Lopes *et al.*, 2007a; Cunha & Almeida, 2009). The highest concentrations

occurred during late spring, in the inner areas, while the lowest occurred during winter, at the entrance of the estuary (Almeida *et al.*, 2002a; Lopes *et al.*, 2007a). Usually, phosphate concentration was higher at low tide (Almeida *et al.*, 2002a). None correlation between salinity and phosphate was observed, but a significant correlation with water temperature was established, defining patterns that were characterized by higher concentrations in spring and summer (Cunha & Almeida, 2009).

The N:P ratio in Ria de Aveiro in the period comprised between October 2000 and June 2001 varied between 7 and 280 (Almeida *et al.*, 2007; Lopes *et al.*, 2007b; Cunha & Almeida, 2009). The average for the whole system during the study period was 40, but ratios are frequently below 16 in summer and autumn (Cunha & Almeida, 2009). The primary production was interpreted as being one of the causes of the low N:P ratio (<16) that prevailed along the Ovar and Mira channels and in the more saline sections of the Ílhavo channel and the Vouga River. Conditions for N limitation of phytoplankton extended throughout early autumn (October) at the outermost sections of all channels and along the Ovar channel with the exception of the inner station (Cunha & Almeida, 2009). The maximum ratio was observed in November–December when rains were heavier, and the lowest was registered in October (Almeida *et al.*, 2007).

1.3.3. PROFILES OF BACTERIAL ABUNDANCE AND ACTIVITY

1.3.3.1. ABUNDANCE

Total bacterial number (TBN) in Ria de Aveiro ranged from 0.2 to 15.3x10^9 cells L^{-1} (Cunha *et al.*, 2000; Almeida *et al.*, 2001a; Almeida *et al.*, 2002b, 2002a; Cunha *et al.*, 2003b; Cunha *et al.*, 2003a; Almeida *et al.*, 2007; Cunha & Almeida, 2009). The longitudinal profile of variation of TBN along the Ílhavo channel fits in a curvilinear pattern with a peak at ~25 to 30 psu (Cunha *et al.*, 2000). Along this channel, TBN ranged from 2.6 to 15.3 x10^9 cells L^{-1}, defining a clear spatial gradient of enrichment towards the inner stations of the mid-estuary, followed by a decline in the transition to riverine station (River Boco) (Cunha *et al.*, 2000). Usually the lowest values of TBN are observed at the mouth of the estuary (station N1) (Almeida *et al.*, 2007) being, on average, 3 times lower than at the inner estuary (station I6) (Almeida *et al.*, 2001a; Almeida *et al.*, 2002b).

The vertical variations of TBN in the estuary are very small, however, perceptible at the deeper zone of the estuary (station N1), where TBN is higher at the top compared with bottom of the water column (Almeida *et al.*, 2001a; Almeida *et al.*, 2002b).

The seasonal variation of TBN in Ria de Aveiro is high, with an overall variation factor of 13.9, averaging 3.5 per sampling station (Almeida *et al.*, 2005). The seasonal variation of TBN in the Ovar channel is similar to Ílhavo channel with highest abundances registered in March–April and lowest in November–December (Almeida *et al.*, 2007). The variation of TBN in Mira and Espinheiro channels was temporarily more stable (Almeida *et al.*, 2007).

The variation of TBN in the estuary is also tidally influenced, with maximum and minimum values occurred around low and high tides, respectively (Almeida *et al.*, 2001a). Compared with the inner section of the estuary (station I6), tidal fluctuation of TBN is stronger at mouth of the estuary, with values 4 times higher at low than at high tide, both at spring and neap tides (Almeida *et al.*, 2002b). In the inner estuary (station I6), where tidal fluctuation was not as obvious as at mouth of the estuary (station N1), TBN was 1.5 times greater at neap than at spring tides (Almeida *et al.*, 2002b).

The particle-attached bacteria (PAB) in Ria de Aveiro varied between 0.02 and 2.5 x 10^9 cells L^{-1} (Almeida & Alcântara, 1992; Almeida *et al.*, 2002b; Cunha *et al.*, 2003b) following, in general, the variation of TBN. The fraction of bacteria attached to particles, averaging 19% of the TBN, is similar in both marine and brackish water zones (range 2– 53%) (Almeida *et al.*, 2002b). Typically, smaller particles were more densely colonized than the larger and, the resuspension of bottom sediments was the main source of PAB in the water column (Almeida & Alcântara, 1992)

1.3.3.2. HETEROTROPHIC ACTIVITIES

The heterotrophic activity of bacterioplankton in the estuarine system Ria de Aveiro is enhanced at the middle and inner sections, in relation to the mouth of the estuary (Cunha *et al.*, 2000; Almeida *et al.*, 2001a; Almeida *et al.*, 2002b; Cunha & Almeida, 2009). The profile of variation of bacterial-dependent activities along one of channels of the estuarine system were in agreement with a curvilinear pattern, peaking at 25 to 30 psu (Cunha *et al.*, 2000). Along the Ílhavo channel, the activity profile revealed a shift from a more N-associated metabolism in the main body of the lagoon to an increasing importance of a C-utilizing community at the mouth of the freshwater stream Rio Boco (Cunha *et al.*, 2000). However, the patterns of variation of heterotrophic activity are distinct in the four channels and in distinct seasonal conditions (Cunha & Almeida, 2009), reflecting different organic matter inputs related to runoff from the margins and salt marshes and to human activities that are considerably distinct between the four estuarine branches (Cunha *et al.*, 2000).

1.3.3.2.1. BACTERIAL BIOMASS PRODUCTION

Bacterial biomass production (BBP) in Ria de Aveiro ranged from 0.05 to 31.0 μg C L^{-1} h^{-1} (Almeida *et al.*, 2001a; Almeida *et al.*, 2002b, 2002a; Almeida *et al.*, 2005; Almeida *et al.*, 2007). Typically, BBP increased towards the inner estuary and the factor of increase was 1.7 from the outer- to the mid-estuary (Almeida *et al.*, 2005) and 3.5 (Almeida *et al.*, 2001a; Almeida *et al.*, 2002b) from the mid- to the inner estuary. BBP in this estuarine system showed a very strong seasonal variation, with an overall factor of 275, averaging 24.8 times per sampling station and being 20 times greater than the overall variation of TBN (Almeida *et al.*, 2005). Frequently, BBP in the estuary peaks in June (75% of the cases) or earlier, in March (25%) (Almeida *et al.*, 2005). However, after a period of intense rain, in a particularly very rainy year, the high inputs of freshwater and allochthonous organic matter, led to significant alteration of BBP seasonal patterns in the estuary, and the maximal values were observed in October or March–April and the minimal in June (Almeida *et al.*, 2007).

BBP in the estuary is also impacted by tidal fluctuation, with maximum and minimum values occur at low and high tides, respectively (Almeida *et al.*, 2001a; Almeida *et al.*, 2002b). The tidal effects on BBP are greater in the entrance compared with inner estuary (Almeida *et al.*, 2001a).

The diel variation of BBP in the estuarine system Ria de Aveiro was investigated as well. During the warm season, BBP decreased approximately 50 % during the night, when compared to diel average. At noon, BP was around twice the diel BBP average while at mid-night, in the same tidal phase, BP was 1.6 times lower than the diel average and 2.7 times lower than the noon value (Almeida *et al.*, 2002a).

At the mouth of the estuary, where the water column is deeper and less turbid, BBP also showed a clear vertical profile of variation at warm season, with average values near the surface being 3.3 times higher than in deep water, but sometimes reached values up to 8.5 times greater. The contribution of the surface water layer to the total BBP at this estuarine zone was 35%. The stratification of BBP was particularly pronounced near low tide. This reaction to surface conditions was not observed in the inner estuary, where BBP values were not significantly different down the water column (Almeida *et al.*, 2001a; Almeida *et al.*, 2002b).

In the inner section of the Ílhavo channel, the transversal variation of BBP was also studied, showing average values 1.5 times greater at the channel margin in 54% of the 24 studied cases (Almeida *et al.*, 2002b).

1.3.3.2.2.EXTRACELLULAR ENZYMATIC ACTIVITY

Planktonic bacterial communities in the estuarine system Ria de Aveiro exhibited generally higher hydrolysis rates for aminopeptidase (Leu-AMPase) than for ß-glucosidase (ß-GlCase) (Cunha *et al.*, 2000). Hydrolysis rates of Leu-AMPase ranged from 179 to 5374 nmol l–1 h–1 and, of ß-GlCase from 0.5 to 489.0 nmol l^{-1} h^{-1} (Cunha *et al.*, 2000; Cunha *et al.*, 2003b; Cunha *et al.*, 2003a; Cunha & Almeida, 2009). Along a salinity gradient the potential for peptide hydrolysis was exceptionally high in the mid-estuary, probably related to intense anthropogenic pressure and increased eutrophication of the brackish water sections of the lagoon (Cunha *et al.*, 2000). ß-GlCase activity increased towards the inner sections of this specific estuarine gradient, reaching its maximum at the freshwater end, the Boco River (Cunha *et al.*, 2000) and showing a significant negative correlation with salinity (Cunha & Almeida, 2009) This different longitudinal profiles of variation of Leu-AMPase and ß-GlCase suggested that the main source of bacterial potential for the degradation of carbohydrates is located upstream, and the major sources of bacterial Leu-AMPase activity in the main body of the lagoon (Cunha *et al.*, 2000).

Hydrolytic activities of bacterioplankton in Ria de Aveiro are influenced by tides, with a general trend of enrichment at low compared with high tide (Cunha *et al.*, 2000; Cunha *et al.*, 2003b). At low tide and in mid-estuary, the hydrolytic activities were up to 4.5 times (Leu-AMPase) and 2.8 times (ß-GLCase) higher than at high tide (Cunha *et al.*, 2000). However, at the freshwater end of the Ílhavo channel, the Boco River, was observed a decrease of the Leu-AMPase activity at low tide to 40% of the high tide value. This decrease was not observed in ß-GlCase activity (Cunha *et al.*, 2000).

The contribution of particle-attached bacteria to total hydrolytic activities in the inner estuary could go from non-existent to 83% in the case of Leu-AMPase and, to 98% in the case of ß-GlCase (Cunha *et al.*, 2003b). Bottom water samples showed maximal percentage of particle-associated ectoenzymatic activity compared with surface. The contribution of attached cells to Leu-AMPase activity showed a slight tendency to decrease during flooding and to regain importance during ebbing. This tidal tendency was not observed for ß-GlCase activity associated to the fraction of particle-attached bacteria (Cunha *et al.*, 2003b).

1.3.3.2.3.INCORPORATION OF MONOMERS

In Ria de Aveiro, incorporation rates of glucose ranged from 0.05 to 57.9 nmol L^{-1} h^{-1} (Cunha *et al.*, 2000; Cunha *et al.*, 2003b; Cunha *et al.*, 2003a) and of leucine from 0 to 25.5 nmol L^{-1} h^{-1} (Cunha & Almeida, 2009). The profiles of monomer incorporation are generally characterized by higher rates at the inner ends of the channels than at the mouth of the estuary. This

pattern was more stable in March and June, when the highest rates of monomer incorporation were observed and also the highest chlorophyll concentrations occurred (Cunha & Almeida, 2009). The longitudinal profile of variation of glucose metabolism along the Ílhavo channel showed major amplifications in the upper mid- or inner-sections, reaching a maximum value at the freshwater end of the channel, at the Boco River (Cunha *et al.*, 2000). The seasonal variation in monomer incorporation was greater than that of ectoenzymatic activities (Cunha & Almeida, 2009).

The heterotrophic metabolism of both glucose and leucine in the estuary showed also a reactive behavior to tidal influence, increasing during ebb and reaching the highest values at low tide (Cunha *et al.*, 2003b; Cunha & Almeida, 2009).

1.3.4. FACTORS OF REGULATION OF BACTERIAL DISTRIBUTION AND ACTIVITY IN THE RIA DE AVEIRO

TBN in Ria de Aveiro is generally influenced by variations of phytoplankton biomass, salinity and temperature (Cunha *et al.*, 2000; Almeida *et al.*, 2001a; Almeida *et al.*, 2002b; Almeida *et al.*, 2007). PAB are negatively influenced by salinity and positively by chlorophyll a (Almeida *et al.*, 2002b; Cunha *et al.*, 2003b), stressing the importance of tidal transport processes of phytoplankton and colonized particles from the low-salinity upper section of the estuary in the regulation of the proportion of attached bacterial cells (Cunha *et al.*, 2003b).

Hydrolytic activities, particularly the activity of Leu-AMPase (>80%), followed a close relation with the variation of bacterial abundance, suggesting that this is a common and widespread hydrolytic capacity of bacterioplankton in the estuary (Cunha *et al.*, 2000). However, the activity of ß-GlCase was strongly associated with the interface, with the limnetic environment being negatively affected by increasing salinity (Cunha *et al.*, 2000).

BBP in Ria de Aveiro was influenced by the absolute size of the active bacteria subpopulation and, in the deeper areas it was also impacted by salinity and depth (Almeida *et al.*, 2001a). The vertical variation of BBP observed at the deep and transparent water column of the estuary mouth, with similar magnitude of the longitudinal variation, was not accompanied by other physicochemical or biological parameters (i.e., total and active bacterial numbers and chlorophyll concentration), which were relatively constant along the water column, suggesting other factors might contribute to stimulate the bacterial activity in this estuarine area. Photochemical transformation of recalcitrant dissolved organic matter into labile compounds may stimulate the growth of active bacteria and explain the activity enhancement near-surface (Almeida *et al.*, 2001a).

In the shallower brackish water zones, only a smaller percentage of variation of BBP was explained by the active bacterial numbers, pointing to the importance of other variables, namely diffusion of nutrients from sediment and allochthonous organic matter inputs for bacteria in this estuarine areas (Almeida *et al.*, 2001a). When the transport of particulate matter through runoff was investigated at ebb tide in the neighborhood of a salt marsh, no evidence was found along the transect for inputs of SPM nor for enrichment in POC or chlorophyll (Almeida *et al.*, 2002b). However, monomer uptake, BBP and protein degradation increased in vicinity of the salt marsh, denoting the presence of stimulation factors (Almeida *et al.*, 2002b; Cunha *et al.*, 2003b). A higher concentration of DOC at the margin of channel, suggest a salt marsh runoff contribution to the channel, which might stimulate the bacterial activity and contribute to the high values observed at mid-estuary (Almeida *et al.*, 2002b).

Bacteria in the Ria de Aveiro also showed a reactive behavior to the environment changing (Almeida *et al.*, 2007). During a particularly heavy rainy year, allochthonous substrates leached out from the surroundings by rain controlled bacterial activity in the estuary, even at the mouth (Almeida *et al.*, 2007). Over an annual time scale, allochthonous substrates exerted a strong influence upon bacterial productivity (37–52% of BBP variation was explained by inorganic nutrient concentration, namely, NO_2^- + NO_3^-). This influence was likely a direct physiological effect on bacterial cells (Almeida *et al.*, 2007). The availability of soluble reactive phosphorus (SRP) and DIN regulate DOM utilization in the Ria de Aveiro in distinct manners, according to the degree of limitation inferred from the DIN:SRP ratios. The general trend corresponds to an increase in polymer hydrolysis with increasing DIN concentration. However, under N-limitation for phytoplankton, the regulation of the initial steps of organic matter recycling is not exerted at the level of polymer hydrolysis but it is directed to monomer incorporation. In these conditions, a negative relation between DIN and amino acid incorporation, as well as the decoupling between hydrolysis and incorporation, emerges (Cunha & Almeida, 2009).

1.4. THESIS OUTLINE

In Ria de Aveiro, bacterial communities have been a target of study in the last twenty years and spatial, seasonal, diel and tidal profiles of activity and abundance have been identify. The main regulator factors were also identified, pointing to the importance of organic and inorganic nutrients to bacteria in the estuarine system. However, the influence of physical processes, such as river discharges and water currents, in the distribution and activity of bacteria is still poorly understood. Although vertical profiles of activity (Almeida *et al.*, 2001a) suggested that photochemical

processes could be important to bacteria in the estuary, this issue remains to be investigated. In order to answer those questions and to improve the knowledge about bacterial distribution and activity in this estuary, with a possible extrapolation to comparable aquatic ecosystems, a number of objectives were outlined:

1.To identify the main factors that influence bacterial activity, abundance and lifestyle under different hydrological conditions in an estuarine environment (chapter 2).

2.To evaluated the influence of the estuarine hydrodynamics in the abundance and productivity of bacterial communities in the SML (chapter 3).

3.To identify patterns of variation and primary environmental regulators of heterotrophic activities of neustonic and planktonic bacterial communities in an estuarine system (chapter 4).

4.To characterize seasonal profiles of variation of colored dissolved organic matter in the estuarine system, as well as evaluate the influence of photochemical and microbial processes in the dynamics of this light absorbing component of DOM in the estuarine system (chapter 5).

To achieve those objectives field surveys as well as microcosms simulations were carried out. Field observations were complemented with the application of numerical models in order understand the hydrodynamics of the estuarine system.

1.4.1.FIELD INVESTIGATIONS AND MICROCOSMS SIMULATIONS

Field investigations were carried out in order to determine the vertical and seasonal profiles of variation of bacterial abundance and heterotrophic activities and their relation with diverse water column properties. Two sites (station N1 and I6) of the estuarine systems Ria de Aveiro (Figure 1.1), with distinct influences of fresh and sea water, hydrodynamics, water properties, amounts and prevailing sources of organic matter and, depth of the water column and transparency were surveyed regularly at the same tidal phase (low tide), approximately every 2 months, during two years. Due to their water column characteristics and location, these two stations are located in lagoon areas currently referred as the marine zone (station N1) and brackish water (station I6) zones (Almeida *et al.*, 2001a; Almeida *et al.*, 2002b).

Station N1, located near the mouth of estuary, in the Canal de Navegação, is highly exposed to oceanic influence and impacted indirectly by the whole freshwater input in the lagoon. The water column depth in this station is 26 m and the average Secchi depth is 1.7 m. The water column becomes stratified as to salinity values under the influence of Vouga River when discharges are higher than 100 m^3 s^{-1} (Vaz *et al.*, 2005; Vaz & Dias, 2008). Tides at this area generate strong

water currents with 1 m s^{-1} (Dias *et al.*, 2000). This site typifies the highly dynamic and unstable conditions of the estuary and, the deep and transparent areas. At this station, fluxes of bacteria, particulate and dissolved materials coming from the brackish sections and/or from the ocean have been observed (Cunha *et al.*, 2003a).

Station I6, located at inner section of the Ílhavo channel, the narrower and shorter of the main channels (Dias *et al.*, 2001), is directly influenced by river Boco discharges and is flanked by salt marshes and mud flats. The water column in vertically homogenous for salinity and it presents an average 2 m depth and 0.7 m secchi depth. Tidal driven water currents at this area are weak (0.5 m s^{-1}) (Dias *et al.*, 2000). This site typifies the low dynamic conditions of the inner estuary and the shallow and turbid areas. At this location land and freshwater sources of bacteria, particulate and dissolved organic and inorganic nutrients have been detected (Almeida *et al.*, 2002b; Henriques *et al.*, 2004).

Samples from SML were collected at both sites with a Plexiglas plate (Harvey & Burzell, 1972), which collects roughly the upper 60–100 µm water layer. Underlaying water was collected with a horizontal Van Dorn bottle at the fixed depths of 20 cm (UW - underlaying water), 50 cm, mid-depth and 50 cm above the sediment surface.

In order to evaluate the influence of photochemical transformations of DOM in the less turbid and deeper areas of the estuary (marine one), microcosm simulations were performed with water samples collected at the N1 station. During these experiments, bacteria-free water samples (0.2 µm filtrate) were irradiated with natural sunlight during long (168 h) and short-term (12 h) periods. Dark controls were included in order to compare with irradiated treatments. Sunlight-induced DOM alterations were evaluated by spectroscopic analysis. After irradiation, sunlight treated and dark controls were inoculated with native bacteria (10-fold diluted 0.7 µm filtrate) and incubated during 168 h. Bacterial responses were evaluated by assessment of abundance and activity.

1.4.2. NUMERICAL MODELS APPLICATION

In order to unveil the importance of hydrodynamics, namely the freshwater inflow, on particle displacement and to assess residence time, simulations were performed setting an average tide situation and varying the freshwater inputs of the five main freshwater tributaries to the Ria de Aveiro, in order to obtain conditions representative of the overall discharge conditions in Ria de Aveiro as well as of the discharges during all the sampling periods. The simulations were performed with the numerical model MOHID (Martins *et al.*, 2001) coupled with a Lagrangian particle-tracking module (Lopes *et al.*, 2006).

In order to reproduce the local hydrodynamics in the areas adjacent to the sampling stations, a previously implemented two-dimensional vertically integrated (2DH) hydrodynamic model for the Ria de Aveiro (Dias & Lopes, 2006b, 2006a) was applied. The water current velocity was obtained by the determination of the root-mean square velocity (V_{rms}) for the areas surrounding the sampling sites.

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CHAPTER 2

2. INFLUENCE OF FRESHWATER INFLOW ON BACTERIAL ABUNDANCE AND ACTIVITY IN THE ESTUARINE SYSTEM RIA DE AVEIRO

Abstract

The influence of freshwater flow on bacterial abundance and activity in the estuarine system Ria de Aveiro (Portugal) was investigated in two sites differently impacted by river inputs and considered representative of the marine (MZ) and brackish water (BZ) zones of the estuary. Sampling events, performed every two months during two-years, were clustered based on hydrological features. The hydrodynamic was simulated with a Lagrangian model and related with microbiological parameters. Estuarine bacterial communities responded to different freshwater regimes developing distinct patterns of abundance and activity at the MZ and BZ. A circulation pattern induced by high river inflow produced vertical stratification at the MZ, promoting a landward flux of phytoplankton and seaward flux of bacterioplankton, and stimulating the import of riverine phytoplankton and particle-attached bacteria to the BZ. Advective transport and resuspension processes contributed to a 3-times increase of the abundance of particle-attached bacteria during intense freshwater inputs. Additionally, bacterial activity in the estuary was controlled by nitrogen concentration, responding to different freshwater inputs, which, in association with different prevailing sources of organic substrates induce significant changes in bacterial production. The dynamics and main controlling factors of bacterial communities in the estuary are clearly impacted by freshwater inputs. Therefore, significant changes on organic and inorganic nutrients recycling by estuarine microbial activities can be expected from alterations in freshwater inputs either related to global climate change or to regional hydrological regimes.

Keywords: bacterial dynamics; particle-attached bacteria; bacterial biomass productivity; freshwater inflow; Lagrangian model; estuary; Ria de Aveiro

2.1. INTRODUCTION

Estuaries are highly dynamic ecosystems, where the mixing between marine and freshwater produces steep gradients of salinity, as well as of organic and inorganic nutrients. Microbial communities adapt to this high variability of physicochemical factors and substrates availability with shifts in activity (Cunha *et al.*, 2000; Almeida *et al.*, 2001b; Cunha *et al.*, 2001; Apple *et al.*, 2008), composition (Crump *et al.*, 1999; Crump *et al.*, 2004; Kirchman *et al.*, 2005; Henriques *et al.*, 2006; Campbell & Kirchman, 2013) or/and lifestyle strategies (Crump *et al.*, 1998; Murrell *et al.*, 1999; Revilla *et al.*, 2000; Karrasch *et al.*, 2003; Selje & Simon, 2003; Lapoussière *et al.*, 2011).

In numerous coastal lagoons and estuaries, water circulation is mainly forced by river flow at the head of the estuary and sea level changes at its mouth (Vaz & Dias, 2008). Even when the water circulation is forced primarily by sea level changes induced by significant tidal ranges, freshwater inflow also determines the general estuarine circulation and thermohaline patterns after periods of strong precipitation (Dias *et al.*, 1999). Additionally, freshwater flow is the predominant source of seasonal and interannual variability in estuaries (Skreslet, 1986), influencing the physics, geology, chemistry, and biology of estuaries through a variety of pathways (Skreslet, 1986; Sklar & Browder, 1998; Almeida *et al.*, 2007). Changes in freshwater flow impact the inundation of the flood plains, advective transport of materials and organisms, dilution or mobilization of contaminants, compression of the estuarine salinity field and density gradient, stratification, and residence time for water (Kimmerer, 2002) and ultimately, estuarine microbial processes.

Although Ria de Aveiro hydrodynamics is tidally dominated (Dias *et al.*, 1999), an exceptional high freshwater input to the estuary, during a particularly rainy year, led to unpredicted seasonal variations of bacterial abundance and activity, stimulated mainly by allochthonous substrates leached from the surroundings (Almeida *et al.*, 2007), reinforcing a reactive behavior of bacterial activity to environmental changes (del Giorgio *et al.*, 2006) and evidencing the multiplicity of environmental factors that controls microbial communities in estuaries (Apple *et al.*, 2008).

This work aims to identify the factors that influence bacterial activity, abundance and lifestyle under different hydrological conditions in an estuarine environment. In order to achieve this goal, two sites with different fresh and sea water influences in the estuarine system Ria de Aveiro were studied and compared, and the influence of rivers inflow on water residence time was assessed by a numerical model application.

2.2. MATERIALS AND METHODS

2.2.1. STUDY SITE

Ria de Aveiro (40º 38'N, 8º 45'W; Figure 2.1) is a shallow tidal lagoon situated on the Northwest Atlantic coast of Portugal, separated from the sea by a sand bar. The lagoon covers an area ranging from 66 to 83 km^2 at low and high tide, respectively. It exchanges with the sea a volume of water of 137 Mm^3 for maximum spring tide and 35 Mm^3 for minimum neap tide (Dias *et al.*, 2000). The lagoon has a complex topography, with four main channels spreading from the mouth, S. Jacinto, Espinheiro, Mira and Ílhavo. Due to their unique characteristics each one could be considering as an independent estuary connected to a common inlet (Dias *et al.*, 2001). Freshwater to lagoon is supplied mainly by rivers Vouga, Antuã, Caster, Gonde and Boco, which discharge an average water input of 1.8 Mm^3 during a tidal cycle (Dias *et al.*, 2003). Of these rivers, the major contributor is Vouga River which discharges more than 66% of the incoming freshwater (Dias *et al.*, 1999) and is connected to the Atlantic Ocean by the Espinheiro Channel. For this study two stations with distinct water column and hydrodynamic characteristics, as well as different impacts of freshwater inflow were selected. Station N1, located near the mouth of estuary, is highly exposed to oceanic influence and impacted indirectly by the whole freshwater input in the lagoon. Station I6, located at inner section of the Ílhavo channel, the narrower and shorter of the main channels (Dias *et al.*, 2001), is directly influenced by river Boco discharge. Due to their water column characteristics and location, these two stations are located in lagoon areas currently referred as the marine zone (MZ) and brackish water (BZ) zones (Almeida *et al.*, 2001a; Almeida *et al.*, 2002a).

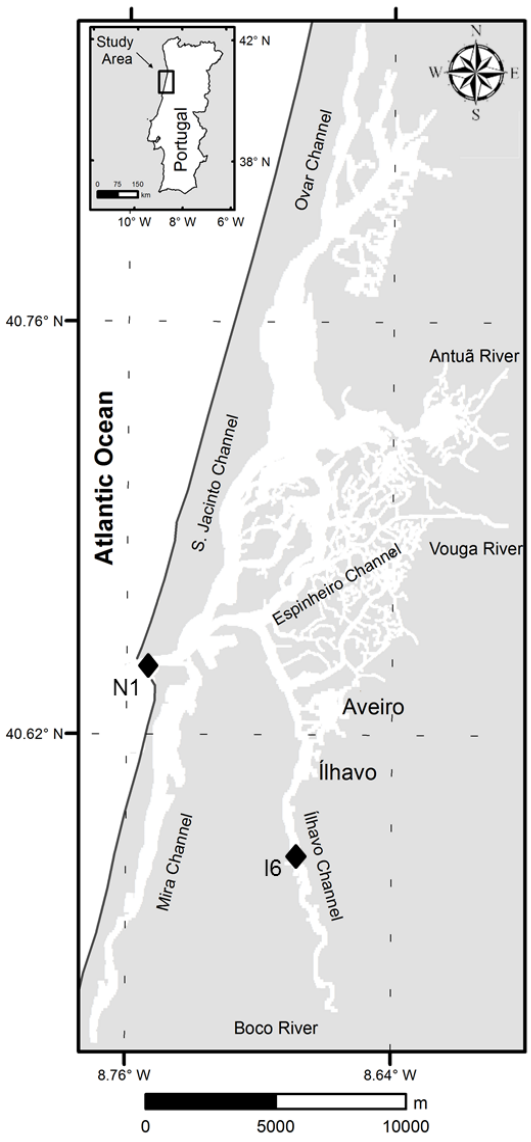


Figure 2.1 The estuarine system Ria de Aveiro with indication of sampling stations. Station N1 in Canal de Navegação represents the marine zone, and station I6, in Canal de Ílhavo, represents the brackish water zone.

2.2.2.SAMPLING

Sampling was conducted at low tide, every two months during two years (2006 and 2007). Water column samples were collected with a Niskin bottle at the fixed depths of 20 cm, 50 cm, mid-column and 50 cm above the sediment surface. Samples were kept at 4 ºC during the transport to the laboratory and processed within 2-3 hours after collection.

2.2.3.METEOROLOGICAL CONDITIONS

Precipitation data prior to sampling events were recorded at the meteorological station of the University of Aveiro. Freshwater inflows values, corresponding to the months of sampling events, were determined by the application of the previous SWAT watershed model, developed in the frame of DyEPlume Project (Table 2-I).

Table 2-I. Monthly average values of flow estimated for rivers Vouga and Boco, and cumulative precipitation of three weeks previous to sampling events recorded at meteorological station of the University of Aveiro.

Year	2006						2007				
Month	Jan	Mar	May	Jul	Sep	Dec	Feb	Apr	Jun	Sep	Dec
Vouga [$m^3 s^{-1}$]	57.7	105.5	34.9	5.4	54.7	222.7	128.8	27.1	69.7	2.7	29.6
Boco [$m^3 s^{-1}$]	3.5	6.8	2.1	0.7	2.3	12.7	7.1	1.7	4.7	0.5	1.7
Precipitation [mm]	65.1	97.0	0.3	0.0	73.2	289.9	124.8	13.5	90.2	0.2	71.6

2.2.4.WATER COLUMN PROPRIETIES

Water temperature and salinity were measured in the field using a WTW LF 196 Conductivity Meter (Wissenschaftlich Technische Werkstätten, Weilheim, Germany). Water column depth was determined with a Sonar probe (Hondex PS-7 LCD Digital Sounder). Secchi depth, as an indicator of water transparency, was determined with a 20 cm Secchi disk.

Chlorophyll a (Chl a) was estimated fluorimetrically (Yentsch & Menzel, 1963) after filtration of 0.5 L triplicate subsamples through Whatman GF/F filters and overnight cold extraction in 90% (v/v) acetone. Suspended particulate matter (SPM) concentration was determined after filtration of triplicate 0.5 L water aliquots through pre-weighted and pre-combusted Whatman GF/F filters. The filters were dried at 60 ºC for 24 h, and SPM was calculated as the increase in dry weight. Particulate organic matter (POM) was determined from the further decrease in weight after 4 h incineration at 550°C (Parsons *et al.*, 1989). For nutrient analysis, water subsamples were filtered through MSI acetate membranes with 0.45 µm pore size and stored at -20 °C in acid-cleaned polyethylene flasks until determination. Orthophosphate and nitrite were quantified using methods described in (Hansen & Koroleff, 2007). Nitrate was assayed using an adaptation of the

spongy cadmium reduction technique (Jones, 1984), with the nitrite value subtracted from the total.

2.2.5. NUMERICAL MODELLING

In this study was used the numerical model MOHID (Martins *et al.*, 2001), which is a three-dimensional baroclinic finite volume model, designed for coastal and estuarine shallow water applications. MOHID solves the three-dimensional incompressible primitive equations, and assumes the hydrostatic equilibrium as well as the Boussinesq and Reynolds approximations. The model equations were presented in several studies and can be consulted in (Vaz, 2007). A previously validated set-up of the MOHID-2D model for the Ria de Aveiro lagoon (Vaz *et al.*, 2007) was applied. Details about the model accuracy to reproduce the local dynamics for this application after calibration and validation are described in Vaz et al (2009).

Furthermore, a Lagrangian particle-tracking module was coupled to the hydrodynamic model, which was used to determine the Lagrangian paths of passive particles released at selected areas of the lagoon (Station N1, Station I6 and mouth of Boco river). Particle displacement is determined from the velocity field computed by the hydrodynamic model and by a random component of velocity (Lopes *et al.*, 2006) and are used as a proxy for water properties at the point of particle emission.

The simulations performed in this study were designed to reveal the importance of the freshwater inflow in particle displacement, aiming to assess their residence time (time they take from Station I6 to leave the lagoon), as well as the time they take from Boco river to Station I6 and from lagoon mouth (Station N1) to Station I6. Thus, three schematic simulations were performed considering always an average tide situation and varying the freshwater input of the five main rivers of the Ria de Aveiro, in order to obtain conditions representative of the overall discharge conditions in Ria de Aveiro as well as of the discharges during all the sampling periods. With this purpose were defined Maximum, Typical and Minimum Discharges Scenarios (Table 2-II). Extreme river discharge values for each scenario were estimated based on the Ria de Aveiro Polis Litoral program, which considered the data presented in the Plano de Bacia Hidrográfica (www.arhcentro.pt) and on the river discharge values available for the sampling periods from a previous application of the SWAT watershed model developed in the frame of DyEPlume Project. The values for the typical discharges were calculated averaging the series values of Ria de Aveiro Polis Litoral program for each river.

Table 2-II Values of River flow into Ria de Aveiro in a scenario maximum, typical and minimum discharges.

River	Minimum Discharges [$m^3 s^{-1}$]	Typical Discharges [$m^3 s^{-1}$]	Maximum Discharges [$m^3 s^{-1}$]
Vouga	11.0	61.3	222.7
Antuã	1.0	4.5	45.0
Cáster	0.8	1.6	38.0
Boco	0.5	1.0	23.0
Ribeira dos Moinhos	1.4	3.0	70.0

2.2.6. TOTAL AND PARTICLE-ATTACHED BACTERIAL NUMBERS

Bacterial cells were enumerated by epifluorescence microscopy using a Leica DMLS microscope equipped with a I 2/3 filter for blue light. Particle-attached cells were counted directly and distinguished from free-living cells on the same slide. Three replicates for each sample were filtered through 0.2 μm black polycarbonate membranes (GE Osmonics) and stained with 0.03 % acridine orange (Hobbie *et al.*, 1977). At least 200 cells or 20 microscope fields were counted for each replicate measurement.

2.2.7. BACTERIAL BIOMASS PRODUCTIVITY (BBP)

BBP was determined in 10-ml triplicate plus a control that was fixed by addition of formaldehyde (2% final concentration). The samples were incubated at a saturating concentration (121.6 nM) of ^{3}H-leucine (Amersham, specific activity - 2.55 TBq $mmol^{-1}$) for 1 h, at *in situ* temperature, in the dark. After incubation, replicates were fixed with 2% (v/v) formaldehyde. Protein was precipitated by the addition of 1 ml of 20% (w/v) ice-cold TCA followed by incubation for 15 min on ice. The 10-ml triplicate and the control were then filtered through 0.2 μm polycarbonate membranes (GE Osmonics) and rinsed with 2 ml of 5% (w/v) ice-cold TCA and 5 ml of 90% (v/v) ice-cold ethanol. Membranes were then placed into 5 mL scintillation vials and 4.5 mL of scintillation cocktail UniverSol (ICN Biomedicals, USA) was added. Radioactivity was measured after a period of 3 days in a Beckman LS 6000 IC liquid scintillation counter. BBP was calculated from leucine incorporation rates using a ratio of cellular carbon to protein of 0.86 and a fraction of leucine in protein of 0.073 (Simon & Azam, 1989).

2.2.8. DATA ANALYSIS

The statistical analysis of data was performed with the SPSS 15.0 (SPSS Statistics) software. Hierarchical cluster analysis was used to outline the main hydrological differences

between the sampling events. The Euclidean distance and Average linkage Between-Groups method was used to clustering and to construct the dendrograms. The significance of the differences observed in microbial parameters among the different hydrological clusters was assessed by One-Way ANOVA. The normality of the data set was confirmed by the Kolmogorov-Smirnov test and the homogeneity of variances was assessed by Levene test. A multiple stepwise linear regression analysis was used to identify the major sources of variability of microbiological descriptors (dependent variables). Physical and chemical parameters were used as independent variables for which autocorrelation were checked.

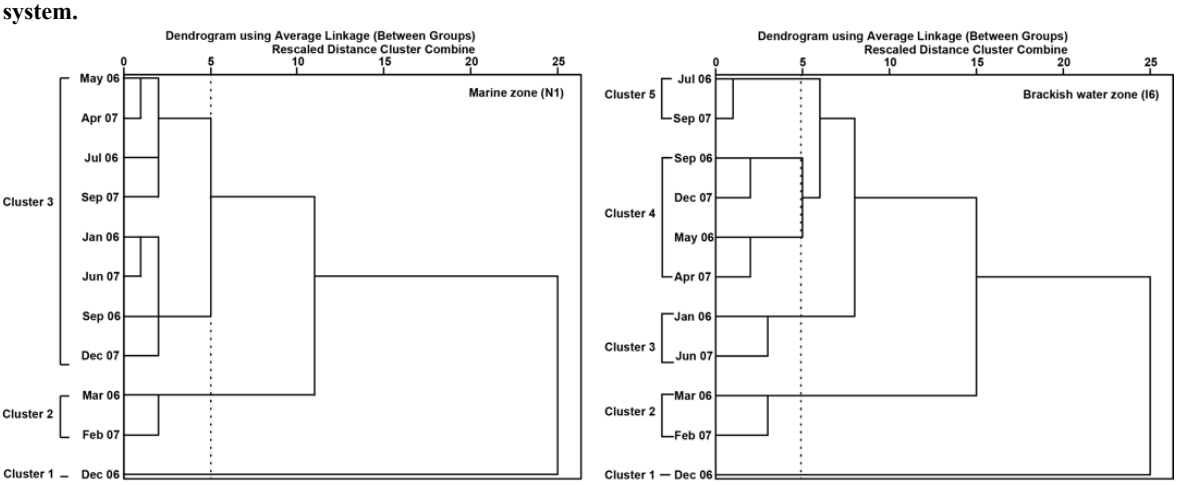
2.3. RESULTS

2.3.1. HYDROLOGICAL CLUSTERS

Salinity, rivers flow and precipitation data obtained during the diverse sampling events (Table 2-I) were clustered using Hieralchical cluster analysis (HCA). The Euclidean distance was used to determine the similarity between-groups and the dendrogram is presented in Figure 2.2. A minimum rescaled distance of 5 was used as criteria for establish clusters individuality.

Due to particularly high inflows of Vouga and Boco rivers, resulting from a previous heavy rain period, the hydrological conditions in December 2006 were distinct from the other sampling events at both MZ and BZ, clustering individually (cluster 1). At the MZ, two more hydrological clusters were distinguished. Cluster 2, comprising the sampling events carried on March 2006, February 2007 and December 2007, and the clusters 3 embracing the events carried in January 2006, September 2006 and June 2007, May 2006, July 2006, April 2007 and September 2007. At the brackish water zone of the estuary, four more individual hydrological clusters were identified. Cluster 2, grouping the events carried in March 2006 and February 2007. Cluster 3 embracing the January 2006, June 2007 and December 2007 sampling events. May 2006, September 2006 and April 2007 events were comprised in the cluster 4, and a particularly salty environment found during July 2006 and September 2007 composed the cluster 5.

Figure 2.2. Dendrograms of the Euclidean distances between sampling events obtained by hierarchical cluster analysis of salinity, precipitation and river flow data at the marine and brackish water zone of the estuarine system.



2.3.2. PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE HYDROLOGICAL CLUSTERS

The physical and chemical characteristics of the water column within the different hydrological clusters at the MZ are presented in Table 2-III and at BZ in Table 2-IV. At the MZ, in December 2006 sampling event (cluster 1), the water column was highly stratified with salinity values ranging from the 10.1 at surface to 35.8 psu at bottom (average 22.2±13.03 psu). At the BZ in this particular event, the water column was homogeneous and presented very low salinity values (average 0.3±0.06 psu). Water temperature in cluster 1 varied between 12.8 and 16.1 ºC (average 14.4±1.62 ºC) at the MZ, and between 10.0 and 10.3 ºC (average 10.1±0.13 ºC) at the BZ. During this extraordinary hydrological situation, the water column was highly turbid and the minimum values of Secchi depth registered at the MZ and BZ were 0.9 and 0.5 m, respectively. At the MZ zone, the sampling events that grouped within cluster 2 presented a stratified water column with salinity values ranging from 23.0 to 34 psu (average 28±5.43 psu) at surface and bottom, respectively. At the BZ, salinity values registered in sampling events of cluster 2 varied between 3.7 and 8.0 psu (average 5.8±2.11 psu). In cluster 2, temperature along the water column varied between 13.9 and 15.5 ºC at the MZ, and between 13.4 and 16.7 ºC at the BZ. Cluster 3 comprises sampling events with average salinity values of 34.7±1.17 psu (32.9 - 36.5 psu) and 18.3±2.81 psu (14.5 – 21.0 psu) at the MZ and BZ, respectively. Water temperature within this specific cluster varied between 12.5 and 19.6 ºC (average 16.2±2.27 ºC) at the MZ and between 10.7 and 19.9 ºC (14.2±4.22 ºC) at the BZ. The average Secchi depth within hydrological cluster 3 was 1.8±0.47 m and 0.8±0.20 m at the MZ and BZ, respectively. At the BZ, in the water column of hydrological cluster 4 the average value of salinity was 27.3±3.59 psu (23.1 – 32.1 psu) and temperature 21.9±1.39 ºC (20.0 - 24.0 ºC). Within cluster 5, salinity values along the water column varied between 35.7 and 36.5 psu (36.2±0.31 psu) and temperature between 18.1 and 24.5 ºC (17.4±4.96

ºC). Average Secchi depth values in the cluster 4 and 5 were 0.8±0.15 m (0.7 – 1.0 m) and 0.6±0.03 (0.6 to 0.7 m).

Table 2-III Properties of the water column within the different clusters, at the marine zone [N1] of the estuarine system Ria de Aveiro.

Marine zone [N1]	Cluster 1	Cluster 2	Cluster 3	Global
Salinity [psu]	22.2±13.03 (N=4) (10.1* – 35.8)	28.3±5.43 (N=8) (23.0 – 34.0)	34.7±1.17 (N=32) (32.9 – 36.5**)	32.4±5.84 (N=44) (10.1 – 36.5)
Temperature [ºC]	14.4±1.62 (N=4) (12.8 – 16.1)	14.6±0.60 (N=8) (13.9 – 15.5)	16.3±2.27 (N=32) (12.5* – 19.6**)	15.8±2.15 (N=44) (12.5 – 19.6)
Chlorophyll a [µg L^{-1}]	2.6±1.34 (N=4) (1.7 – 4.6)	3.3±0.97 (N=8) (1.9 – 4.6)	2.9±1.69 (N=32) (0.6* – 6.0**)	3.0±1.54 (N=44) (0.6 – 6.0)
SPM [mg L^{-1}]	83.8±71.66 (N=4) (26.1* – 178.1**)	50.5±15.49 (N=8) (34.4 – 80.3)	61.8±10.29 (N=32) (44.1 – 76.7)	61.8±23.29 (N=44) (26.1 – 178.1)
POM [mg L^{-1}]	17.3±12.86 (N=4) (6.9* – 33.7**)	13.6±3.74 (N=8) (9.5 – 20.5)	14.9±4.55 (N=32) (9.9 – 24.5)	14.8±5.44 (N=44) (6.9 – 33.7)
DOC [mg L^{-1}]	1.0±0.41 (N=4) (0.5 – 1.4)	5.6±3.33 (N=8) (1.2 – 9.8)	3.5±2.96 (N=32) (0.1* – 13.9**)	3.7±3.09 (N=44) (0.1 – 13.9)
NO_3^- + NO_2^- [µM]	12.4±6.44 (N=4) (6.5 – 19.4**)	5.4±2.64 (N=8) (2.2* – 10.3)	6.8±3.24 (N=32) (2.9 – 12.8)	7.1±3.85 (N=44) (2.2 – 19.4)
PO_4^{3-} [µM]	26.9±9.58 (N=4) (16.2 – 38.6**)	1.4±0.84 (N=8) (0.8 – 3.4)	1.3±0.70 (N=12) (0.1* – 3.0)	3.7±7.89 (N=44) (0.1 – 38.6)
Secchi depth [m]	0.9* (N=1)	1.8 (N=1)	1.8±0.47 (N=8) (1.2 – 2.6**)	1.7±0.51 (N=10) (0.9 – 2.6)

average ± standard deviation (minimum – maximum);
* Lowest value observed;
**Highest value observed;
SPM - suspended particulate matter; POM - particulate organic matter; DOC - dissolved organic carbon;

Table 2-IV. Water column characteristics within the different clusters in the brackish water zone [I6] of the estuarine system Ria de Aveiro.

Brackish water zone [I6]	Cluster 1	2	3	4	5	Global
Salinity [psu]	0.3±0.06 (N=4) (0.2* – 0.3)	5.8±2.11 (N=8) (3.7 – 8.0)	18.3±2.81 (N=12) (14.5 – 21.0)	27.3±3.59 (N=12) (23.1– 32.1)	36.2±0.31 (N=8) (35.7 – 36.5**)	20.1±12.01 (N=44) (0.2 – 36.5)
Temperature [ºC]	10.1±0.13 (N=4) (10.0* – 10.3)	15.1±1.69 (N=8) (13.4 – 16.7)	14.2±4.22 (N=12) (10.7 – 19.9)	21.9±1.39 (N=12) (20.0 – 24.1)	21.3±3.19 (N=8) (18.1 – 24.5**)	17.4±4.96 (N=44) (18.1 – 24.5**)
Chlorophyll a [µg L^{-1}]	3.2±0.18 (N=4) (3.0 – 3.4)	5.0±0.86 (N=8) (4.0 – 6.5)	2.6±1.11 (N=12) (1.1* – 3.9)	7.1±2.75 (N=12) (3.3 – 10.1**)	4.3±2.33 (N=8) (2.0 – 7.6)	4.6±2.51 (N=44) (1.1 – 10.1)
SPM [mg L^{-1}]	29.7±0.79 (N=4) (28.5* – 30.2)	43.3±6.50 (N=8) (35.5 – 50.7)	62.2±26.21 (N=12) (29.3 – 91.3)	67.1±10.71 (N=12) (45.2 – 83.1)	87.1±20.06 (N=8) (67.9 – 116.8**)	61.8±23.76 (N=44) (28.5 – 116.8)
POM [mg L^{-1}]	5.4±0.15 (N=4) (5.3* – 5.6)	9.2±1.97 (N=8) (7.1 – 11.6)	15.5±8.20 (N=12) (7.3 – 31.2**)	15.3±2.75 (N=12) (9.4 – 19.7)	17.9±2.83 (N=8) (11.9 – 20.4)	13.8±6.02 (N=44) (5.3 – 31.2)
DOC [mg L^{-1}]	4.3±2.35 (N=4) (2.1* – 7.6)	9.7±5.81 (N=8) (2.1 – 19.2)	13.3±6.08 (N=12) (3.8 – 23.3)	9.3±2.08 (N=12) (7.3 – 12.8)	13.5±10.10 (N=8) (2.4 – 26.0**)	10.9±6.66 (N=44) (2.1 – 26.0)
NO_3^- + NO_2^- [µM]	13.7±7.02 (N=4) (4.3 – 21.2)	67.2±23.06 (N=8) (33.2 – 92.3**)	19.7±10.89 (N=12) (3.0* – 53.0)	23.5±5.55 (N=12) (10.0 – 29.9)	15.0±7.06 (N=12) (3.2 – 24.7)	28.0±24.07 (N=44) (3.0 – 92.3)
PO_4^{3-} [µM]	46.2±4.77(N=4) (41.0 – 50.4)	2.7±1.09 (N=8) (1.4 – 4.0)	1.4±0.56 (N=12) (0.7 – 2.5)	2.1±1.67 (N=12) (0.4 – 5.3)	3.1±0.47 (N=8) (2.5 – 3.9)	5.3±11.44 (N=44) (0.4 – 50.4)
Secchi depth [m]	0.5* (N=1)	0.5 (N=1)	0.8±0.20 (N=3) (0.7 – 0.9)	0.8±0.15 (N=3) (0.7 – 1.0**)	0.6±0.03 (N=2) (0.6 – 0.7)	0.7±0.17 (N=10) (0.5 – 1.0)

average ± standard deviation (minimum – maximum);
* Lowest value observed;
** Highest value observed;
SPM - suspended particulate matter; POM - particulate organic matter; DOC - dissolved organic carbon;

2.3.3. INFLUENCE OF FRESHWATER INFLOW IN THE RESIDENCE TIME OF WATER MASSES

The application of the hydrodynamic numerical model to simulate a scenario of maximum freshwater inflow in the estuarine system, similar to the conditions of the cluster 1, showed a very low residence time of water masses in the estuary. Particles released from the mouth of the estuary under this hydrological condition were rapidly flushed out and, therefore it was not possible to represent their trajectory. When released from the station I6, particles are rapidly transported along

the Ílhavo channel, reaching the mouth of the estuary within 55-66 h (Figure 2.4-B). Under this extreme scenario, particles released from the Boco River reach station I6 after 25-30 h (Figure 2.4-C). The simulation of a typical situation of medium values of freshwater inflow to the system, similar to those of the clusters 3, showed a higher residence time of water in the Ílhavo channel. During this typical scenario, particles released from the entrance of the estuary reached the station I6 after 75-90 h (Figure 2.5-A) and remaining in the channel after 240-288 h when released from the station I6 (Figure 2.5-B). Particles released from Boco River under a medium inflow of freshwater reached the station I6 only after 175-215 h (Figure 2.5-C). During a minimum freshwater inflow situation particles released from the mouth of estuary reached the station I6 after 60-68 h (Figure 2.6-A). During this extreme situation, such as found during the events of cluster 5, particles released from the station I6 remain in upper zone of the Ílhavo channel after 360-432 h (Figure 2.6-B) and reached the station I6 after 288-300 h when realized from the Boco River (Figure 2.6-C).

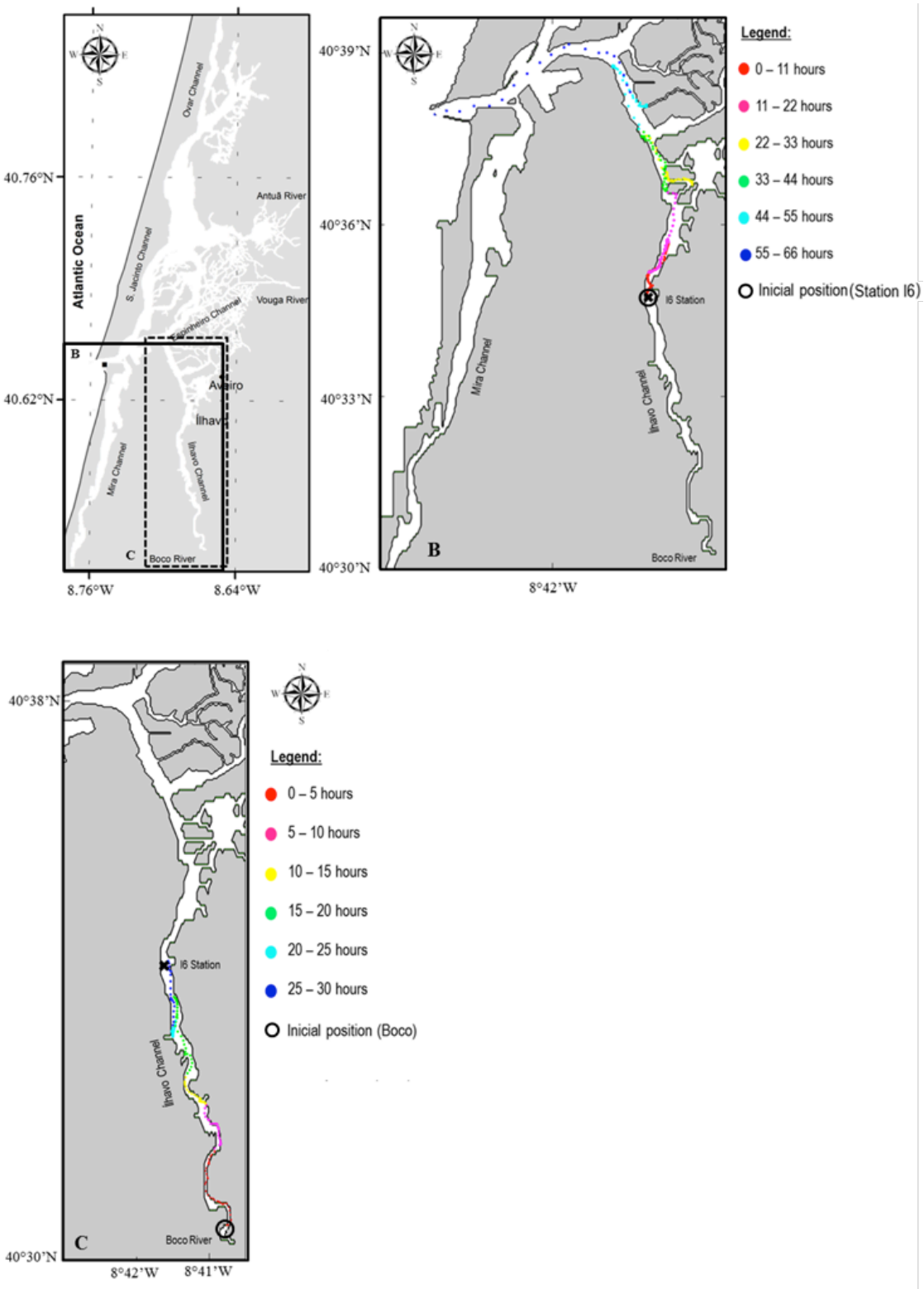


Figure 2.4. Time evolution of particles trajectory released from station I6 (B) and Boco River (C) under maximum influence of river inflow in the estuarine system Ria de Aveiro.

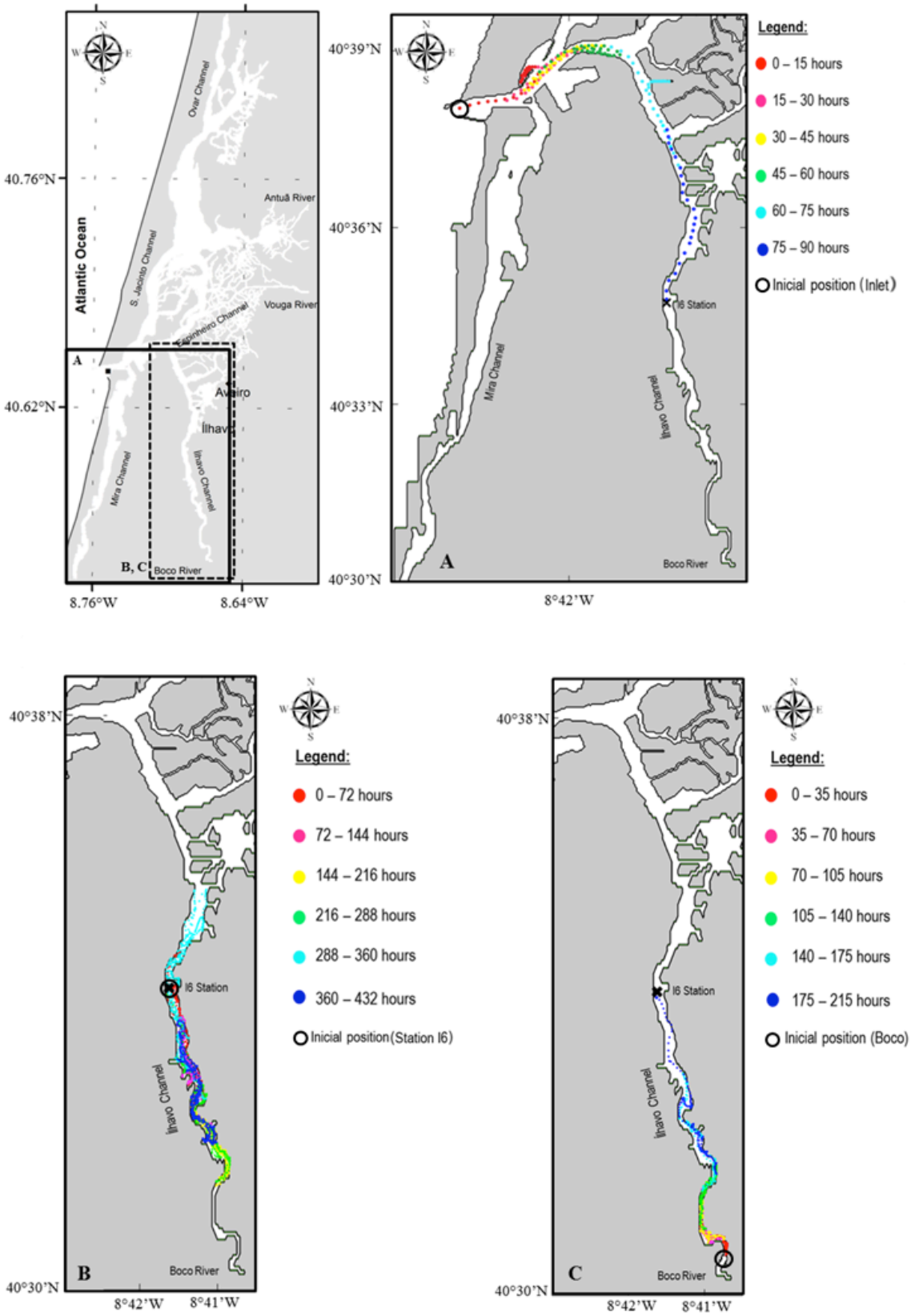


Figure 2.5. Time evolution of particles trajectory released from the mouth of the estuary (A), the station I6 (B) and Boco River (C) in a typical situation of freshwater inflow in the estuarine system Ria de Aveiro.

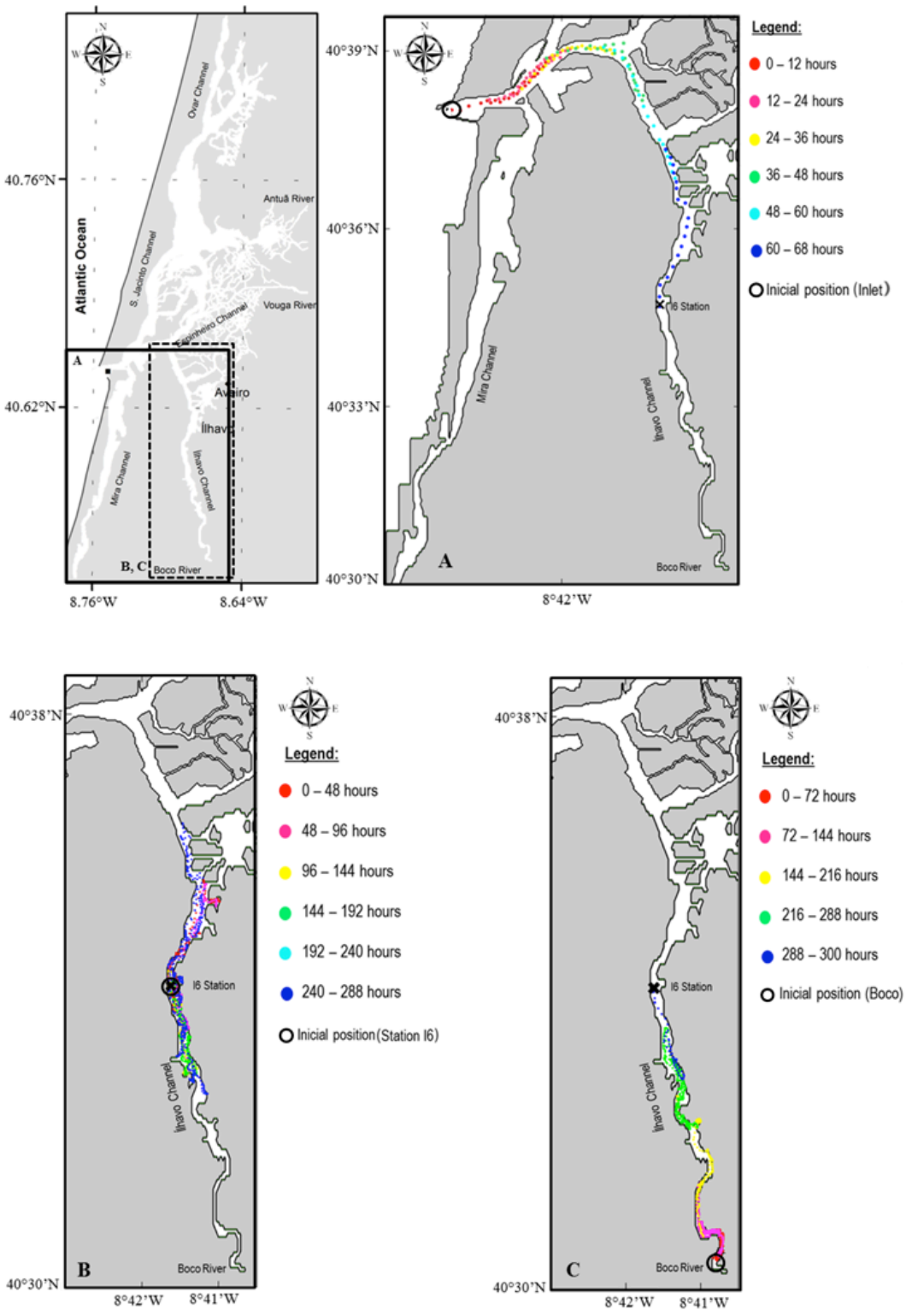


Figure 2.6. Time evolution of particles trajectory released from the mouth of the estuary (A), the station I6 (B) and Boco River (C) under minimal influence of river inflow in the estuarine system Ria de Aveiro

2.3.4. CHLOROPHYLL A, PARTICULATE MATTER, DISSOLVED ORGANIC CARBON AND NUTRIENTS

Concentrations of chlorophyll a, particulate matter, dissolved organic carbon and nutrients in the water column of the different hydrological clusters at the MZ and BZ are shown in Table 2-III and 2-IV, respectively. The concentration of chlorophyll a at the MZ was similar in the different hydrological clusters (ANOVA, $p>0.05$), varying between 0.6 and 6.0 µg L^{-1} (average 3.0±1.54 µg L^{-1}). At the BZ, the concentration of chlorophyll a in the hydrological cluster 4 was 1.7, 2.2 and 2.7 times higher (ANOVA, $p<0.05$) than in clusters 5, 1 and 3, respectively. The average concentration of chlorophyll a in this estuarine zone was 4.6±2.51 µg L^{-1} (1.1 – 10 µg L^{-1}). The concentration of suspended particulate matter (SPM) in the water column of the MZ was on average, 61.8±23.29 mg L^{-1} and similar in the different clusters (ANOVA, $p>005$). SPM concentration within the hydrological cluster 5 at BZ zone was 2.0 and 2.9 times higher (ANOVA, $p<0.05$) than in the clusters 2 and 1, respectively. The average SPM of the water column at this estuarine zone was 61.8±23.76 mg L^{-1}. The concentration of particulate organic matter (POM) at the MZ was similar in the different hydrological clusters (ANOVA, $p>0.05$), varying between 6.9 and 33.7 mg L^{-1} (average 14.8±5.44 µg L^{-1}). At the BZ, POM concentration in the water column ranged from 5.3 to 31.2 mg L^{-1} (13.8±6.02 mg L^{-1}) and was 1.9 and 3.3 times higher (ANOVA, $p<0.05$) in hydrological cluster 5, in relation to clusters 2 and 1, respectively. Average dissolved organic carbon (DOC) concentration at the MZ was 3.7±3.09 mg L^{-1} and in cluster 2, it was 2.1 and 5.6 times higher (ANOVA, $p<0.05$) than in clusters 3 and 1, respectively. At the BZ, average concentration of DOC in the water column was 10.9±6.66 mg L^{-1}. Within the hydrological cluster 5, DOC concentration was 3.1 times higher (ANOVA, $p<0.05$) than in the cluster 1. The average concentration of $NO_3^- + NO_2^-$ was 7.1±3.85 µM at the MZ and 28.0±24.07 µM at the BZ. At the MZ, the concentration of $NO_3^- + NO_2^-$ in cluster 1 was significantly higher (ANOVA, $p<0.05$) than in other hydrological clusters. At the BZ, the concentration of $NO_3^- + NO_2^-$ within the hydrological cluster 2 was significant higher (ANOVA, $p<0.05$) than in other clusters. PO_4^{3-} concentration was on average, 3.7±7.89 µM at the MZ and 5.3±11.44 µM at the BZ. The concentration of PO_4^{3-} in the cluster 1 was 20 times significantly higher (ANOVA, $p<0.05$) than in the other cluster at the two zones.

2.3.5. TOTAL AND PARTICLE-ATTACHED BACTERIA

Total abundance of bacteria (Table 2-V; Figure 2.3) at the MZ was similar (ANOVA, $p>0.05$) in the different hydrological clusters, varying between 0.3 and 7.6 × 10^9 cells L^{-1} (average 2.2±1.98 x 10^9 cells L^{-1}). At the BZ, total bacterial numbers ranged from 1.4 to 13.0 × 10^9 cells L^{-1} (average 3.9±2.39 x 10^9 cells L^{-1}) and were 2.1 times higher (ANOVA, $p<0.05$) in cluster 5, compared with the cluster 3. The abundance of bacteria attached to particles (Table 2-V; Figure 2.3)

varied between 0.3 and 15.0 × 10^8 cells L^{-1} at the MZ and between 0.2 to 13.0 ×10^8 cells L^{-1} at the BZ. At MZ and BZ, particle-attached bacteria numbers in the water column of sampling events with high inputs of freshwater (clusters 1 and 2) were, on average, 3 times higher (ANOVA, $p<0.05$) than in the other hydrological conditions. The fraction of particle-attached bacteria (Table 2-V) at the MZ ranged from 2.2 to 71.4 %, decreasing from the cluster 1 to cluster 3, as the influence of rivers diminishes. At the BZ, the fraction of bacteria attached to particles varied between 1.3 and 44.8 % and the maximum value was observed within the hydrological cluster 2.

Table 2-V. Variation of total bacterial number (TBN), particles-attached bacteria (PAB), fraction of particle-attached bacteria (%PAB) and bacterial biomass production (BBP) in the water column, within the different clusters at the marine [N1] and brackish water [I6] zones of the estuarine system Ria de Aveiro.

	Cluster					Global
	1	2	3	4	5	
			Marine zone [N1]			
TBN [x10^9 cells L^{-1}]	1.4±0.35 (N=4) (1.0 – 1.9)	2.1±1.83 (N=8) (0.5 – 4.7)	2.3±2.14 (N=32) (0.3* – 7.6**)			2.2±1.98 (N=44) (0.3 – 7.6)
PAB [x10^8 cells L^{-1}]	6.4±4.81 (N=4) (2.1 – 13.0)	5.7±5.04 (N=8) (0.5 – 15.0**)	1.6±0.89 (N=32) (0.3* – 3.5)			2.8±3.22 (N=44) (0.3 – 15.0)
PAB %	41.4±21.64 (N=4) (21.3 – 71.4**)	28.2±12.35 (N=8) (10.1 – 54.0)	10.6±7.19 (N=32) (2.2* – 32.2)			16.8±14.37 (N=44) (2.2 – 71.4)
BBP [µg C L^{-1} h^{-1}]	6.1±2.50 (N=4) (3.8 – 9.7)	4.8±2.07 (N=8) (1.9 – 8.3)	9.0±8.65 (N=32) (0.6* – 32.1**)			7.9±7.62 (N=44) (0.6 – 32.1)
			Brackish water zone [I6]			
TBN [x10^9 cells L^{-1}]	4.6±0.31 (N=4) (4.2 – 4.9)	3.5±1.16 (N=8) (1.7 – 5.0)	2.9±1.47 (N=12) (1.4* – 6.2)	3.5±0.95 (N=12) (2.0 – 4.7)	6.2±4.52 (N=8) (1.6 – 13.0**)	3.9±2.39 (N=44) (1.4 – 13.0)
PAB [x10^8 cells L^{-1}]	9.2±2.76 (N=4) (7.1 – 13.0**)	9.4±1.37 (N=8) (7.4 – 11.0)	3.0±1.73 (N=12) (0.3 – 6.0)	5.0±2.14 (N=12) (2.5 – 8.1)	3.1±2.63 (N=8) (0.2* – 8.6)	5.3±3.29 (N=44) (0.2 – 13.0)
PAB %	20.0±4.76 (N=4) (16.4 – 27.0)	29.1±8.31 (N=8) (20.8 – 44.8**)	11.3±6.65 (N=12) (2.0 – 21.3)	13.9±4.00 (N=12) (6.4 – 21.6)	5.8±4.64 (N=8) (1.3* – 16.5)	15.1±9.54 (N=44) (1.3 – 44.8)
BBP [µg C L^{-1} h^{-1}]	10.0±2.05 (N=4) (8.0 – 12.6)	9.0±8.95 (N=8) (2.8 – 26.8**)	12.1±8.93 (N=12) (2.6* – 26.3)	9.3±2.60 (N=12) (6.4 – 15.9)	12.8±6.37 (N=8) (7.2 – 21.8)	10.7±6.68 (N=44) (2.6 – 26.8)

DP – Dependent variable; Adj. r^2 – Adjusted r^2; TBN – Total bacterial number; PAB – particles-attached bacteria; %PAB – fraction of particles-attached bacteria; BBP – bacterial biomass production; Sal – salinity; Temp – temperature; Chl a – Chlorophyll a; SPM- suspended particulate material; POM – particulate organic matter;

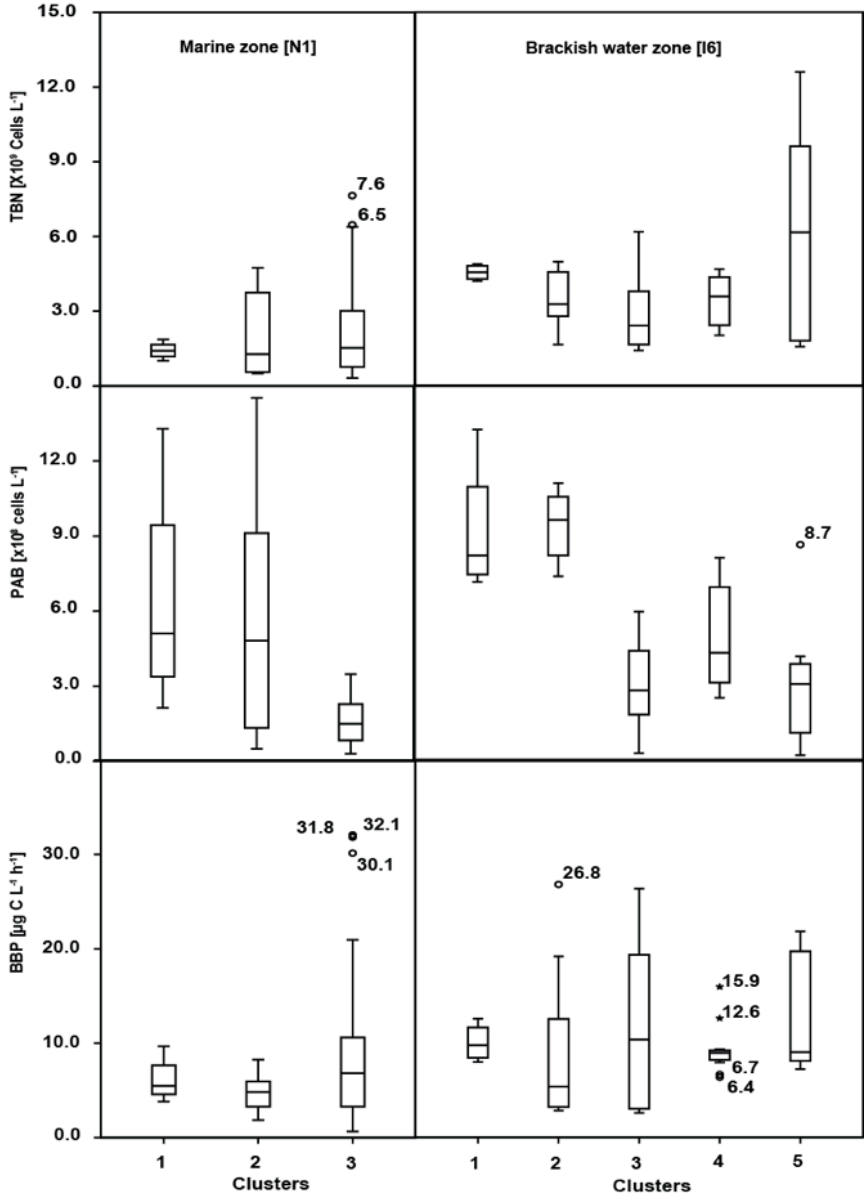


Figure 2.3. Box plots of the total bacterial numbers (TBN), particle-attached bacteria (PAB), and of bacterial biomass production (BBP) for each of the different hydrological conditions at the marine [N1] and brackish water [I6] zones of estuarine system Ria de Aveiro.

2.3.6. BACTERIAL BIOMASS PRODUCTIVITY (BBP)

BBP (Table 2-V; Figure 2.3) at the MZ was on average 7.9 ± 7.62 µg C L^{-1} h^{-1} (0.6 – 32.1 L^{-1} h^{-1}), increasing from the hydrological cluster 2 to 3 as the influence of rivers diminishes. The observed differences were not statistically significant (ANOVA, $p>0.05$). BBP at the BZ water zone was similar between hydrological conditions (ANOVA, $p>0.05$), varying between 2.6 and 26.8 µg C L^{-1} h^{-1} (average 10.7 ± 6.68 µg C L^{-1} h^{-1}).

2.3.7. STEPWISE MULTIPLE REGRESSION ANALYSIS

The results of stepwise multiple regression analyses are presented in Table 2-VI. At the MZ, the independent variables related to primary production and suspended particulate matter explained the variability of total and particle-attached bacterial numbers along the water column within the different hydrological clusters. Bacterial activity at this particular estuarine zone was explained by the concentration of inorganic nutrients or by SPM depending on the hydrological conditions. At the BZ, dissolved organic matter, salinity and water temperature explained the variability of total bacterial numbers in the diverse hydrological clusters. Chlorophyll a concentration and phosphate concentration explained the variability of bacteria attached to particles at this particular estuarine zone. Here, inorganic nitrogen, organic matter and water temperature explained bacterial activity variation in the water column depending on hydrological conditions.

Table 2-VI. Regression equations for the variation of microbiological parameters along the water column within the different clusters obtained from stepwise multiple regression analysis.

DP	Cluster	Independent variables	Regression equation	Adj. R^2
		Marine zone		
TBN	2	Chl a (ß=-0.899; p=0.002)	Log TBN = 10.5 – 0.405 Chl a	0.776
	3	Chl a (ß=0.557; p=0.002)	Log TBN = 8.76 + 0.132 Chl a	0.284
PAB	2	Chl a (ß=-1.269; p=0.001) SPM (ß=0.551; p=0.038)	PAB = $1.84x10^9$ - $6.63x10^8$ Chl a + $1.79x10^7$ SPM	0.870
	3	Not explained by the set of independent variables		
% PAB	2	SPM (ß=0.768; p=0.026)	%PAB = -2.71+0.61 SPM	0.522
	3	Temp (ß=-0.645; p=0.000) PO_4^{3-} (ß=-0.367; p=0.028)	%PAB = 52.89 – 2.31 Temp – 4.40 PO_4^{3-}	0.393
BBP	2	NO_3^-+NO_2^-(ß=-0.838;p=0.009)	BBP = 8.32 – 0.66 NO_3^- + NO_2^-	0.652
	3	PO_4^{3-} (ß=-0.539; p=0.001) SPM (ß=0.403; p=0.009)	BBP = -2.79 – 8.01 PO_4^{3-} + 0.34 SPM	0.467
		Brackish water zone		
TBN	2	DOC (ß=0.880; p=0.004)	Log TBN = 9.283 + 0.24 DOC	0.775
	3	Sal (ß=0.951; p=0.000) Temp (ß=-0.414; p=0.008)	Log TBN = 8.41 + 0.70 Sal – 0.02 Temp	0.874
	4	Sal (ß=0.660; p=0.001) Temp (ß=0.434; p=0.009)	Log TBN = 8.00 + 0.24 Sal + 0.04 Temp	0.848
	5	DOC (ß=0.978; p=0.000)	Log TBN = 9.14 + 0.38 DOC	0.950
PAB	2	Not explained by the set of independent variables		
	3	Chl a (ß=0.723; p=0.008)	PAB = $1.54x10^8$ + $1.13x10^8$ Chl a	0.475
	4	PO_4^{3-} (ß=0.915; p=0.000)	PAB = $2.53x10^8$ + $1.17x10^8$ PO_4^{3-}	0.821
	5	Chl a (ß=0.823; p=0.012)	PAB = $-8.35x10^7$ + $9.30x10^7$ Chl a	0.624
% PAB	2	Chl a (ß=0.902; p=0.002)	%PAB = 14.64 + 8.75 Chl a	0.782
	3	POM (ß=0.700; p=0.011)	%PAB = 2.51 + 0.57 POM	0.439
	4	PO_4^{3-} (ß=0.697; p=0.012)	%PAB = 10.35 +1.66 PO_4^{3-}	0.434
	5	*Not explained by the set of independent variables*		
BBP	2	NO_3^-+NO_2^-(ß=-0.787;p=0.020)	BBP = 29.54 – 0.31 NO_3^-+NO_2^-	0.557
	3	POM (ß=0.899; p=0.000)	BBP = -3.71 + 0.98 POM	0.789
	4	Temp (ß=-0.650; p=0.022)	BBP = 36.04 – 1.22 Temp	0.365
	5	NO_3^-+NO_2^- (ß=0.708; p=0.001) DOC (ß=-0.521; p=0.003)	BBP = 7.69 + 0.64 (NO_3^-+NO_2^-)- 0.33 DOC	0.939

2.4. DISCUSSION

2.4.1. PATTERN OF VARIATION OF BACTERIAL ABUNDANCE AND ACTIVITY IN RELATION TO HYDROLOGIC CONDITIONS

In Ria de Aveiro, the pattern of variation of total bacterial abundance and activity in relation to freshwater inputs was different at the MZ and BZ. At the MZ, total bacterial numbers increased as the influence of freshwater decreased, but at the BZ, bacterial abundance showed a concave parabolic pattern of variation, with high abundances at the extreme hydrological regimes, and lower at the transitional situations between wet and dry seasons. Bacterial biomass production at the MZ decreased from cluster 1 to 2, increasing to maximum values within the cluster 3 whereas, at the BZ, the average values were similar within the different clusters, with no clear trend related with the hydrological regime. However, the pattern of variation of particle-attached bacteria was similar at both estuarine zones, with high abundances during periods of high freshwater inputs into the estuarine system. When river discharges decreased to medium levels the number of bacteria associated to particles decreased 4 and 3 times in MZ and BZ, respectively. A similar sharp temporal decline of particles-attached bacteria has been also observed in San Francisco estuary and was related with a decrease of the intensity of physical processes resulting from reduction of freshwater influence (Murrell *et al.*, 1999).

2.4.2. RELATION BETWEEN HYDROLOGY AND PHYTOPLANKTON AND BACTERIOPLANKTON DYNAMICS

The two studied sampling sites have distinct hydrological characteristics resulting from their location and different exposure to oceanic and riverine influences. Reflecting the local hydrological features, under the influence of high freshwater inputs in the estuary (cluster 2), the concentration of chlorophyll a (a proxy to phytoplankton biomass) related positively or negatively with salinity depending on the location. Moreover, the factors that influence bacterioplankton abundance at the different estuarine zones were also different. At MZ, bacterioplankton variation was explained by chlorophyll a whereas, at BZ, was explained by DOC.

Located at the mouth of the estuary, the MZ is highly exposed to oceanic influence, but during winter and spring seasons it is also impacted by river discharges, particularly the Vouga River, the main freshwater source of the estuarine system (Dias *et al.*, 1999). When Vouga river flow is higher than 100 $m^3\ s^{-1}$, such as cluster 1 and 2, a typical salty stratified water column is generated, with a characteristic freshwater outflow at the top and a marine water inflow at the bottom (Vaz *et al.*, 2005; Vaz & Dias, 2008). Under this dynamic, phytoplankton biomass was positively related with salinity (Adj. R^2=0.506; ß=0.742; p=0.006; N=12), which in association

with a stratified water column indicated a phytoplankton enrichment in the bottom salty water layer. Bacterioplankton in turn, showed a negative correlation with phytoplankton, indicated a bacterial enrichment at the top of water column. This different enrichment of bacterio- and phytoplankton, allied to the typical dynamic of a stratified water column, suggested opposite fluxes of phytoplankton and bacterioplankton cells, following the inflow of bottom salty water and the outflow of the top brackish water layer, respectively. An oceanic import of inorganic nutrients, chlorophyll a and DOC, followed by an increase of primary production was previously observed in this estuarine system (Almeida *et al.*, 2005), as well as a net export of bacterial cells, with estimations of net fluxes for a tidal cycle ranged from −26.0 to -2.5×10^{16} bacterial cells (Cunha *et al.*, 2003a). The balance between marine and freshwater inputs to the main body of the estuary during each tidal cycle determines the magnitude of the exports from the estuary to the sea and reflects the total size and activity of estuarine communities (Cunha *et al.*, 2003a).

At the BZ, under the influence of high Boco River flow (cluster 2), the water column was homogenous with low salinity values (average 5.8 psu) and chlorophyll a related negatively with salinity (Adj. R^2=0.691; ß=-0.857; p=0.006; N=8), indicating a riverine source of phytoplankton biomass. Previous studies determined that phytoplankton specific growth rate in this estuarine system during the cold season, corresponding to the period of high freshwater inputs, ranged from 0.02 to 0.12 d^{-1} (Almeida *et al.*, 2002a) and, therefore, doubling times vary between 5.8 and 34.7 d. The numerical modelling simulation showed that, under the high influence of Boco River, particles move rapidly along the Ílhavo channel, reaching the station I6 only 25-30 h after releasing at the river mouth. The advection time is clearly shorter than the doubling time of phytoplankton biomass, supporting evidence of a freshwater source of primary production at this estuarine site. The variation of bacterial abundance at this particular estuarine zone, during this hydrological regime, was explained by the concentration of DOC, which in turn showed a strong negative correlation with chlorophyll a (Adj. R^2=0.641; ß=-0.832; p=0.010; N=8) supporting previous observations (Almeida *et al.*, 2005) concerning the importance of non-phytoplanktonic carbon sources to estuarine bacterial growth, particularly at the inner sections.

2.4.3. FACTORS INFLUENCING PARTICLE-ATTACHED BACTERIA

In estuaries, the different pattern of spatial and temporal distribution suggest that free and attached bacteria are not under the control of the same physical processes (Painchaud *et al.*, 1995b). A clear freshwater influence on the dynamics of particle-attached bacteria in Ria de Aveiro is indicated by an increased number and respective fraction of bacteria attached to particles at both estuarine zones during high riverine discharges.

During a high influence of freshwater water (cluster 2) the abundance of particle-attached bacteria at the MZ was explained by the variation of chlorophyll a and SPM. The similar inverse relation between total and particle-attached bacteria and phytoplankton, suggest analogous seaward flux of bacteria attached to particles in the stratified water column. Additionally to the vertical density gradients promoted by the freshwater outflow, the positive correlation with SPM concentration supports previous observations that the resuspension of bottom sediments is the main factor governing the frequency of colonised particles in Ria de Aveiro (Almeida & Alcântara, 1992) and reinforces importance of also short-term local processes as sediment resuspension in controlling the abundance of particle-attached bacteria in the estuaries (Painchaud *et al.*, 1995b).

During periods of high discharge of River Boco, particle-attached bacteria numbers at the BZ could not be explained by the set of the studied variables, but their proportion showed a direct relation with the concentration of chlorophyll a. A temporal divergence between the maximum abundance of particle-attached bacteria (clusters 1 and 2) and phytoplankton biomass (cluster 4), suggest their direct relationship came from a similar allochthonous freshwater source, sharing similar transport processes induced by riverine discharges and not by direct influence. The numerical modelling simulation showed that residence time of water masses in the Ílhavo channel under the influence of high inputs of Boco River is low, and particles released from the river mouth reached this particular estuarine zone only after 25-30 h and the mouth of the estuary after 55-66 h. A previous study determined that doubling time of bacteria in the estuary, during the cold season, corresponding to the period of high inputs of freshwater, and within similar tidal conditions (low tide) ranged between 1.1 e 2.2 days (Almeida, personal communication). Thus, doubling time of bacteria is higher than advection time supporting an evidence of a freshwater source of particle-attached bacteria. (LaMontagne & Holden, 2003) observed that distinct particle-associated bacteria have been detected in estuaries sampled during periods of high flow (Bidle & Fletcher, 1995; Crump *et al.*, 1999), but little differences between particles associated and free-living fractions has been reporting during summer, low-flow periods (Hollibaugh *et al.*, 2000).

Therefore, the dynamics of particle-attached bacteria in Ria de Aveiro is highly impacted by advective transport induced by freshwater inflow and resuspension processes. The intensity of these physical processes might determine as well the degree of differentiation between free and particles-attached bacterial communities. We propose a conceptual model of the dynamics of phytoplankton as well as total and particle-attached bacteria in this estuarine system under the influence of high freshwater inputs (Figure 2.7).

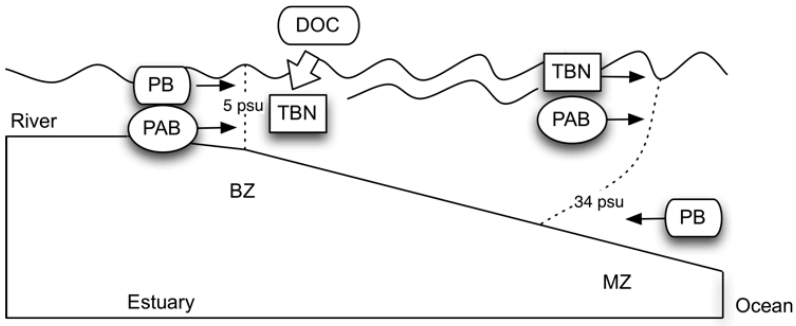


Figure 2.7. Model of the impact of high freshwater inputs on the dynamics of phytoplankton biomass (PB), total (TBN) and particle-attached bacteria (PAB) at the marine (MZ) and brackish water (BZ) zones of the estuarine system Ria de Aveiro. The present study indicates that, under a high influence of freshwater, the stratified water column dynamics at MZ promote an estuarine importation of PB and exportation of both TBN and PAB. At the BZ, the rapid advection of water masses promotes a riverine supply of PB and PAB.

2.4.4. FACTORS INFLUENCING BACTERIAL ACTIVITY

The activity of bacteria in Ria de Aveiro is impacted by freshwater inputs. Under the influence of high inputs of freshwater (cluster 2), bacterial biomass production showed an inverse relationship with the concentration of NO_3^- + NO_2^- at both MZ and BZ of the estuary. A negative relation between N availability and leucine incorporation in Ria de Aveiro has been previously reported, with a probable utilisation of leucine as alternative N source under N-limitation to phytoplankton. In this condition, a negative relation between DIN and amino acid incorporation emerges, and the decoupling between hydrolysis of polymers and incorporation of monomers occurs (Cunha & Almeida, 2009). Although stoichiometric concentrations of N and P suggested possible N-limitation of phytoplankton in cluster 2 at the MZ, resulting from a high phytoplankton growth, the high concentration of NO_3^- + NO_2^- observed at the BZ clearly indicates that nitrogen was in excess and phytoplankton here might experienced P instead of N limitation. Therefore, the similar inverse response of MZ and BZ bacterial activities to NO_3^- + NO_2^- suggest that high concentrations of DIN supplied by the high inputs of freshwater, inhibits bacterial biomass production in the estuarine system Ria de Aveiro, perhaps by inhibition of amino acid incorporation (Cunha & Almeida, 2009), but others factors than nutrient stoichiometry that covariate with freshwater might regulate this limitation.

Contrastingly, when freshwater influence decreased to minimum values at the BZ, bacterial biomass production was positively related with NO_3^- + NO_2^-. This relation emerges with an unexpected inverse correlation with DOC, considering that its highest values were observed during this period of extremely low discharges of Boco River. Therefore, other factors than the amount of DOC might regulate the activity of BZ bacteria under this hydrological regime. The application of the Lagrangian model showed that water masses during this dry period have a long residences time, with particles remaining in the inner estuary after 18 days. Moreover, the relative low concentration

of chlorophyll a, the lowest values of NO_3^- + NO_2^- and its stoichiometry with PO_4^{3-} suggested that phytoplankton growth was N-limiting during this regime. A combination of a low *in situ* primary production with a long residence time of water might promote the exhaustion of more labile substrates, traditionally nitrogen-rich organic compounds (Hopkinson *et al.*, 1998) and the DOC measurements did not reflect the changes in the quality of the organic matter. The utilization of the remaining nitrogen-poor organic compounds might result in an increased reliance on inorganic nitrogen for bacterial growth demands (Caron, 1994; Kirchman, 1994). Additionally, nutrient-limited phytoplankton could produce predominantly high C:N and high C:P organic compounds which, when utilized by the bacteria, result in competition by the bacteria for limiting nitrogen or phosphorus (Caron, 1994).

The positive correlation between bacterial biomass production and NO_3^- + NO_2^- support a bacterial activity requirement of DIN uptake under this hydrological regime, fomented by a high concentration of nitrogen-poor organic compounds, resulting from the exhaustion of nitrogen rich substrates and/or phytoplankton production under N-limited conditions. In environments with high inputs of organic matter with a high C:N ratio, high bacterial turnover rates, and high concentrations of nitrate relative to preferred substrates such as ammonium and amino acids a significant bacterial assimilation of nitrate is expected (Middelburg & Nieuwenhuize, 2000).

Our results support previous observations of inorganic regulation of heterotrophic activity in the estuarine system Ria de Aveiro (Almeida *et al.*, 2007; Cunha & Almeida, 2009) and provide evidences of both high and low inorganic nitrogen concentrations, resulting from the seasonal variations of freshwater inputs, impacted bacterial biomass production. Although the mechanisms involved remain to be clarified in further investigations, eventually by microcosms experiments, we propose a conceptual model of bacterial biomass regulation by DIN (Figure 2.8). Bacterioplankton metabolism may not respond directly to freshwater flow *per se* but to variables that covary with freshwater flow (Murrell *et al.*, 1999)

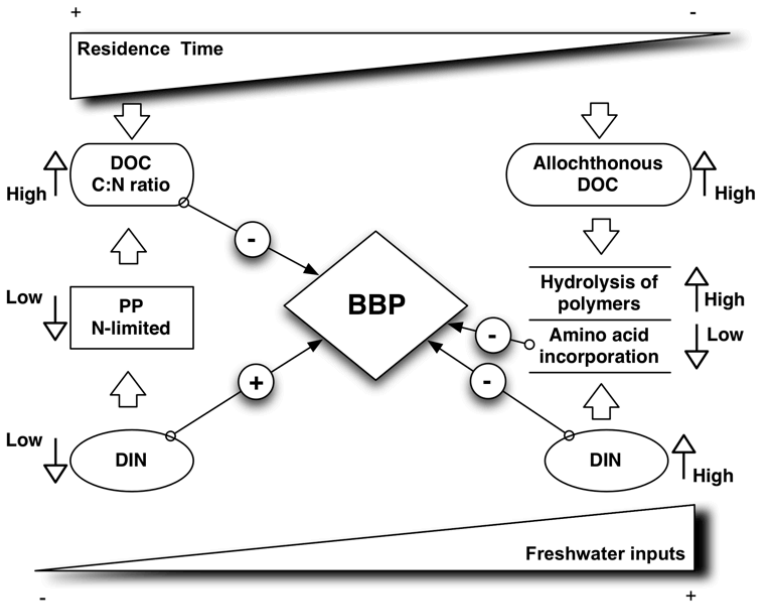


Figure 2.8. Model of the effect of freshwater inputs on bacterial biomass production (BBP) in the estuarine system Ria de Aveiro. The present study indicates that the different inorganic nitrogen concentration, resulting from the different seasonal freshwater inputs and prevailing organic sources in the estuarine system produces an inhibition or stimulation of bacteria biomass production. DOC – dissolved organic matter; PP- primary production; DIN- dissolved inorganic nitrogen; ⊕ - stimulation; ⊗ - inhibition;

2.5. CONCLUSION

Even in tidal dominated estuarine systems, such as Ria de Aveiro, seasonal shifts in freshwater inputs produces significant direct and indirect alterations on the dynamics, lifestyle and activity of bacteria. Our study shows that patterns of variation of estuarine bacterial abundance and activity vary according the hydrological regime and the extension of local riverine or/and oceanic impacts. High freshwater inputs increase the abundance of particle-attached bacteria in the estuary and induce significant changes in the dynamics of both phyto- and bacterioplankton. Hydrological features, subsequent to high inputs of freshwater in the estuary, promote oceanic phytoplankton imports and estuarine bacterioplankton exports at the MZ, and import of riverine phytoplankton and of bacteria associated to particles at BZ. Furthermore, the activity estuarine bacteria are controlled by different nitrogen concentrations, resulting from different freshwater inputs, which in association with different prevailing sources of organic substrates induce significant changes on bacterial biomass production. The present work improved the knowledge about the impact of freshwater inputs on estuarine bacterial communities, allowing anticipation of possible outcomes on estuarine biogeochemical cycles resulting from changes in freshwater inputs, as a consequence of climate alterations or/and regional freshwater management.

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CHAPTER 3

3. RELATION BETWEEN BACTERIAL ACTIVITY IN THE SURFACE MICROLAYER AND ESTUARINE HYDRODYNAMICS

Abstract

Bacterial communities of the surface microlayer (SML) of the estuary Ria de Aveiro (Portugal) were characterized in terms of abundance and activity during a 2-year survey at two sites with distinct hydrodynamic properties (marine and brackish water zones). The hydrodynamic conditions were simulated using a bidimensional numerical model and related to the microbiological observations. The pattern of variation of bacterial biomass productivity (BBP) was distinct between the two sampling sites. At the outer site, BBP was significantly lower at the SML, whereas at the inner site, it was significantly enhanced at the SML. Although the total bacterial abundance was similar in the SML and underlying water (UW), the fraction of cells attached to particles was significantly higher at the SML (two to three times). The integration of microbiological results with environmental and hydrological variables shows that strong currents in the marine zone promote the vertical mixing, inhibiting the establishment of an SML bacterial community distinct from that of UW. In contrast, in the brackish water zone, lower current velocities provide conditions for enhancing the bacterial activity in the enriched SML. Estuarine dynamics influence the distribution and activity of microorganisms at the SML and in the water column, with anticipated impacts for the carbon cycle in the estuarine.

Keywords: surface microlayer; bacterioneuston; bacterioplankton; hydrodynamic model; Ria de Aveiro.

3.1. INTRODUCTION

The surface microlayer (SML) is a ubiquitous feature in aquatic environments (Henk, 2004) and represents the interface between the hydrosphere and the atmosphere, being operationally defined as the uppermost millimeter of the water column (Liss & Duce, 1997). Several physical and biological processes, including simple diffusion, turbulent mixing, rising bubbles, in situ primary production, convection and upwelling of underlying waters (UW) (Liss & Duce, 1997), contribute to the enrichment of organic and inorganic nutrients as well as microorganisms at this interface. Physically and chemically distinct from the UW (Zhang *et al.*, 1998; Zhang *et al.*, 2003), the SML is considered a high-stress extreme environment for microorganisms (Lion & Leckie, 1981), where sudden transitions in temperature and salinity occur and the organisms are exposed to intense solar radiation, namely in the UV range.

Bacteria are key players in organic matter recycling in the aquatic ecosystems, mediating the flux of nutrients and energy to higher trophic levels (Cho & Azam, 1988). The SML is a temporally and spatially variable microbial habitat (Stolle *et al.*, 2010), offering a combination of both favorable and detrimental factors. Enhanced (Hardy, 1982; Kuznetsova *et al.*, 2004; Agogué *et al.*, 2005; Aller *et al.*, 2005; Hörtnagl *et al.*, 2010) and reduced (Obernosterer *et al.*, 2005; Joux *et al.*, 2006; Obernosterer *et al.*, 2008; Cunliffe *et al.*, 2009) bacterial abundances at SML compared with UW have been reported for different aquatic ecosystems. The variability of the enrichment factors (EF) (ratio abundance at the SML/abundance in UW) of bacteria at SML might reflect the heterogeneities of the systems studied (Agogué *et al.*, 2004).

The activity of bacteria might reflect the balance between the growth-promoting and growth-inhibiting processes occurring at SML (Obernosterer *et al.*, 2005). Generally considered a net heterotrophic habitat (Sieburth *et al.*, 1976; Obernosterer *et al.*, 2005), the values for descriptors of bacterial activity at SML diverge between aquatic ecosystems. Enhanced (Sieburth *et al.*, 1976; Obernosterer *et al.*, 2005), similar (Agogué *et al.*, 2004) or reduced (Dietz *et al.*, 1976; Joux *et al.*, 2006; Obernosterer *et al.*, 2008) heterotrophic bacterial activity in relation to bulk water has been reported for the air–water interface.

Estuaries represent a challenging environment where physical, chemical and biological processes are closely coupled. Water circulation is one of the major drivers of biological processes in an estuary (Day *et al.*, 1989). The hydrodynamic regime determines vertical mixing, water residence time, light penetration as a result of suspended materials dynamics, salinity fields and nutrient availability (Zimmerman, 1988; Kjerfve & Magill, 1989) and ultimately the microbiological processes. This work aimed to test, by the application of a numerical model, whether the hydrodynamics of the estuarine system Ria de Aveiro can explain the differences in bacterial abundance and productivity between SML and UW. Two sites with distinct

hydrodynamics and water properties were studied and compared, and the influence of water current velocity on microbial dynamics at the air–water interface was evaluated.

3.2. MATERIALS AND METHODS

3.2.1. STUDY SITE

Ria de Aveiro (40°38′N, 8°45′W; Figure 3.1) is a shallow tidal lagoon situated on the Northwest Atlantic coast of Portugal, separated from the sea by a sand bar. The lagoon covers an area of 66 and 83 km^2 at low and high tide, respectively. It exchanges with the sea a volume of water of 137 Mm^3 for maximum spring tide and 35 Mm^3 for minimum neap tide (Dias *et al.*, 2000). Several rivers carry fresh water into the lagoon with an average water input of 1.8 Mm^3 during a tidal cycle (Dias *et al.*, 2003). The lagoon has a complex topography, with different channels spreading from the mouth in diverse streams, forming a complex estuarine system. For this study, two stations with distinct geo- and hydrodynamic characteristics were selected. Station N1, located near the mouth of the estuary, in an area currently referred to as the marine zone, is characterized by a deep, clear and salty water column. It is highly exposed to winds and tidal currents, as well as circulation of ships, and presents unstable conditions for the establishing of a distinct SML. Station I6, located in the inner estuary, usually referred to as the brackish water zone, is characterized by a shallow, turbid and productive water column (Almeida *et al.*, 2001a; Almeida *et al.*, 2002b). The lower hydrodynamics and the distance from shipping activities allow more stable conditions for the establishment of a distinct SML.

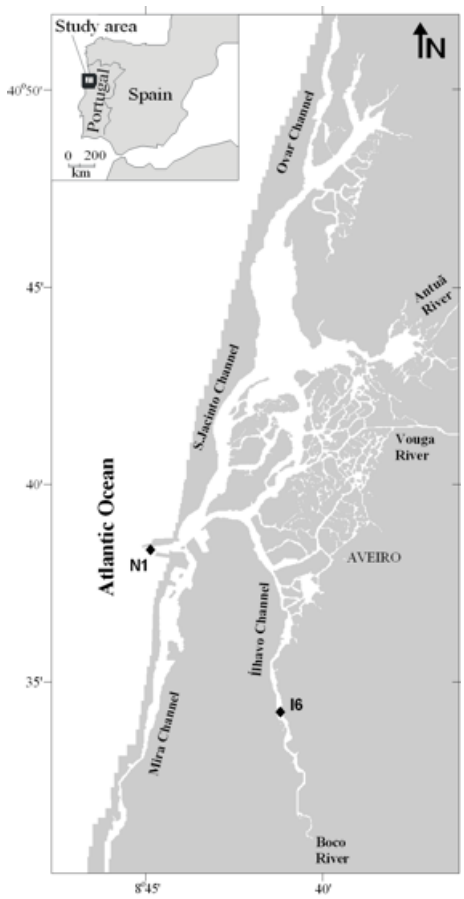

Figure 3.1. The estuarine system Ria de Aveiro with indication of sampling stations. Station N1 in Canal de Navegação represents the marine zone, and station I6, in Canal de Ílhavo, represents the brackish water zone.

3.2.2. METEOROLOGICAL CONDITIONS

Wind speed data concurrent with the sampling were recorded at the meteorological station of the University of Aveiro, located close to the sampling area.

3.2.3. SAMPLING

Sampling was conducted at low tide, every 2 months during 2006 and 2007. SML samples were collected using a Plexiglas plate (Harvey & Burzell, 1972), which collects roughly the upper 60–100 μm water layer. The plate dimensions were 0.25 m wide × 0.35 m long and 4 mm thick. Before sampling, the plate was cleaned with ethanol, rinsed with sterile distilled water and finally with water from the sampling site. The water adhering to the plate during immersion was removed from both sides by introducing the plate between two Teflon sheets and collecting the water into a sterilized glass bottle. Samples from UW were collected using a Niskin bottle at 20 cm depth and transferred to a sterilized glass bottle. Samples were kept cold during the transport to the laboratory and processed within 2–3 h of collection.

3.2.4. PHYSICAL AND CHEMICAL VARIABLES

Temperature and salinity were measured in the field using a WTW LF 196 Conductivity Meter (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany), and dissolved oxygen concentration was measured using a WTW OXI 320 oxygen meter (Wissenschaftlich-Technische Werkstätten). Temperature and dissolved oxygen concentration were only determined for UW, because the collection of the SML is a slow process during which changes in temperature and oxygen concentration may occur.

3.2.5. NUMERICAL MODELLING

The Ria de Aveiro is a shallow lagoon, with its dynamics essentially dominated by the oceanic tidal propagation along its channels. Accordingly, a two-dimensional vertically integrated (2DH) hydrodynamic model was chosen to predict tidally induced velocities in the areas adjacent to the sampling stations.

A numerical model with these characteristics and developed from the SIMSYS2D model (Leendertse & Gritton, 1971; Leendertse, 1987) was applied to the data. It consists of a numerical code that solves a set of finite difference equations resulting from the discretization of the well-known shallow water equations. The alternating direction implicit method is applied using a space-staggered grid (Leendertse & Gritton, 1971; Dias *et al.*, 2001) to solve those difference equations.

The model results consist of sea surface elevation and instantaneous horizontal velocities averaged over the entire depth of the water column for the full computational domain. This model

was previously implemented for the Ria de Aveiro using the respective numerical bathymetry (square grids with dimensions of 100 m) and the imposition of proper boundary and initial conditions. It was also calibrated and validated against field data (Dias & Lopes, 2006b, 2006a) and proved to accurately reproduce the local hydrodynamics.

In this investigation, we predicted the instantaneous velocities for all cells of the numerical bathymetry with a time step of 40 s, for a complete tidal cycle centered in each sampling period.

To obtain a representative velocity for the sampling locations at each period, we determined the root-mean square velocity (V_{rms}) for the areas surrounding the sampling sites (Figures 3.2 and 3.3) from instantaneous velocity values (V) determined for the full tidal cycles through the application of the equation:

$$V_{rms} = \left(\frac{1}{N} \sum_{i=1}^{N} V^2 \right)^{1/2}$$

where N is the total number of velocity values for each cell in a full tidal cycle (N=1118).

3.2.6. TOTAL AND PARTICLE-ATTACHED BACTERIAL NUMBERS

Bacterial cells were enumerated by epifluorescence microscopy using a Leica DMLS microscope equipped with an I 2/3 filter for blue light. Particle-attached cells were counted directly and distinguished from free-living cells on the same slide. Three replicates for each sample were filtered through 0.2-µm black polycarbonate membranes (GE Osmonics) and stained with 0.03% acridine orange (Hobbie *et al.*, 1977). At least 200 cells or 20 microscope fields were counted for each replicate measurement.

3.2.7. BACTERIAL BIOMASS PRODUCTIVITY (BBP)

BBP was determined in a 10-mL triplicate plus a control that was fixed by the addition of formaldehyde (2% final concentration). The samples were incubated at a saturating concentration (121.6 nM) of ^{3}H-leucine (Amersham, specific activity – 2.55 TBq mmol^{-1}) for 1 h, at in situ temperature, in the dark. After incubation, replicates were fixed with 2% v/v formaldehyde. Protein was precipitated by the addition of 1 mL of 20% w/v ice-cold trichloroacetic acid (TCA), followed by incubation for 15 min on ice. The 10-mL triplicate and the control were filtered through 0.2-µm polycarbonate membranes (GE Osmonics) and rinsed with 2 mL of 5% w/v ice-cold TCA and 5 mL of 90% v/v ice-cold ethanol. Membranes were then placed in 5-mL scintillation vials and 4.5

mL of scintillation cocktail UniverSol (ICN Biomedicals) was added. Radioactivity was measured after a period of 3 days in a Beckman LS 6000 IC liquid scintillation counter. BBP was calculated from leucine incorporation rates using a ratio of cellular carbon to protein of 0.86 and a fraction of leucine in protein of 0.073 (Simon & Azam, 1989).

3.2.8. DATA ANALYSIS

The microbiological parameters determined for SML and UW were compared and expressed as the EF defined as $EF=[X]_{SML}/[X]U_W$, where [X] is the concentration of a given parameter (GESAMP, 1995). The statistical analysis of microbiological data was performed using the SPSS 15.0 (SPSS Statistics) software. The relations between the different parameters were examined using a Spearman correlation. The significance of the differences observed in microbial parameters between the two layers was assessed using one-way ANOVA.

3.3. RESULTS

3.3.1. PHYSICAL AND CHEMICAL PARAMETERS

In the marine water zone (N1), the average salinity values were 29.1 ± 8.89 psu (range – 11.0–36.6 psu) at SML and 28.5 ± 8.70 psu (range – 10.1–36.3 psu) in the UW. In the brackish water zone (I6), the average salinity values were 20.0 ± 12.35 psu (range – 0.3–36.8 psu) at SML and 19.9 ± 12.39 psu (range – 0.2–36.5 psu) in UW (Table 3-I). Water temperature had a seasonal profile of variation, ranging from 12.7 to 19.4 °C (average – 16.0 ± 2.55 °C) and from 10.3 to 24.4 °C (average – 17.5 ± 5.21) in the station N1 and station I6, respectively (Table 3-I). Dissolved oxygen concentrations (Table 3-I) ranged from 2.5 to 11.4 mg L^{-1} (average – 7.8 ± 3.46 mg L^{-1}) in the marine zone (station N1) and from 1.9 to 12.3 mg L^{-1} (average – 6.4 ± 3.51 mg L^{-1}) in the brackish water zone (station I6). Secchi depth (Table 3-I) varied between 0.90 and 2.62 m (average – 1.7 ± 0.51 m) in the station N1 and between 0.46 and 1.00 m (average – 0.7 ± 0.17 m) in the station I6 (Table 3-I).

Table 3-I. Physical and chemical parameters determined in the marine (N1) and brackish water (I6) zones of the estuary Ria de Aveiro during the sampling events.

Year	2006						2007				
Month	Jan	Mar	May	Jul	Sep	Dec	Feb	Apr	Jun	Sep	Dec
Marine zone [N1]											
Salinity [psu]											
SML	34.0	23.6	34.9	36.5	35.9	11.0	23.3	34.4	33.1	36.6	17.3
UW	32.9	23.3	34.2	36.3	35.5	10.1	23.2	33.9	33.2	33.2	17.2
Temperature [ºC]											
UW	12.7	15.0	18.2	18.2	19.4	13.2	14.0	19.0	17.4	16.0	13.0
Oxygen [mg L^{-1}]											
UW	10.5	9.1	5.3	ND	2.7	2.5	9.7	10.9	ND	8.3	11.3
Secchi depth [m]	1.22	ND	2.33	1.88	2.62	0.90	1.82	1.36	1.55	1.83	1.93
Brackish water zone [I6]											
Salinity [psu]											
SML	20.8	7.9	27.3	36.8	30.0	0.3	3.8	23.5	19.6	35.8	14.6
UW	20.7	7.4	26.9	36.5	30.3	0.2	3.7	23.1	19.5	35.9	14.5
Temperature [ºC]											
UW	11.0	16.7	24.1	24.4	22.1	10.3	13.5	20.6	19.8	18.2	11.8
Oxygen [mg L^{-1}]											
UW	12.3	5.9	3.4	ND	1.9	2.2	7.9	7.9	ND	6.5	9.7
Secchi depth [m]	0.93	ND	0.66	0.65	0.70	0.46	0.48	0.74	0.65	0.60	1.00

ND, Not determined

3.3.2. METEOROLOGICAL CONDITIONS

During the sampling events, wind speed varied between 1.0 and 40.3 m s^{-1} (average – 13.1 ± 12.44 m s^{-1}) and was eight times more intense during 2006 compared with 2007 (Table 3-II).

Table 3-II. Daily mean of wind speed during the sampling events in Aveiro (Portugal)

Year	2006						2007				
Month	Jan	Mar	May	Jul	Sep	Dec	Feb	Apr	Jun	Sep	Dec
Wind intensity [m s^{-1}]	11.4	40.3	27.3	18.4	17.0	15.7	1.0	2.0	4.9	4.0	1.6

3.3.3. CURRENT VELOCITY

The numerical model for the estuarine system showed that the two sampling sites are hydrodynamically distinct. At the entrance of the estuary (station N1, Figure 3.2), the estimated intensities of currents ranged from 0.50 to 1.36 m s^{-1} (average 0.97 m s^{-1}) and were, on average, 4.4 times higher than those in the inner site. In this estuarine section (station I6, Figure 3.3), the velocity of currents varied between 0.14 and 0.27 m s^{-1} (average 0.22 m s^{-1}).

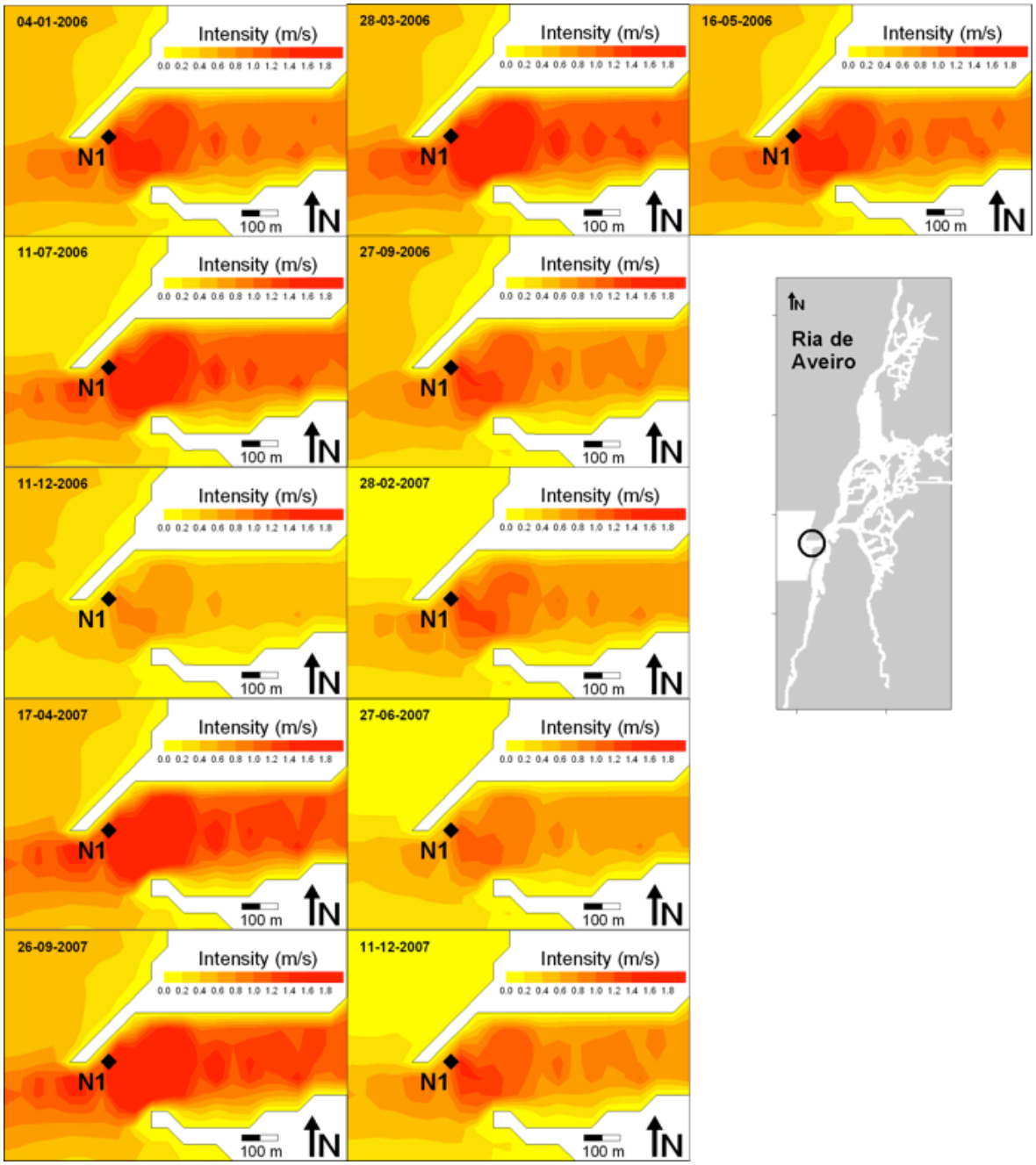

Figure 3.2. Root-mean square velocity field determined from instantaneous numerically predicted velocity values for a complete tidal cycle centered in each sampling period for the more hydrodynamic station (marine zone – N1) of the estuarine system Ria de Aveiro.

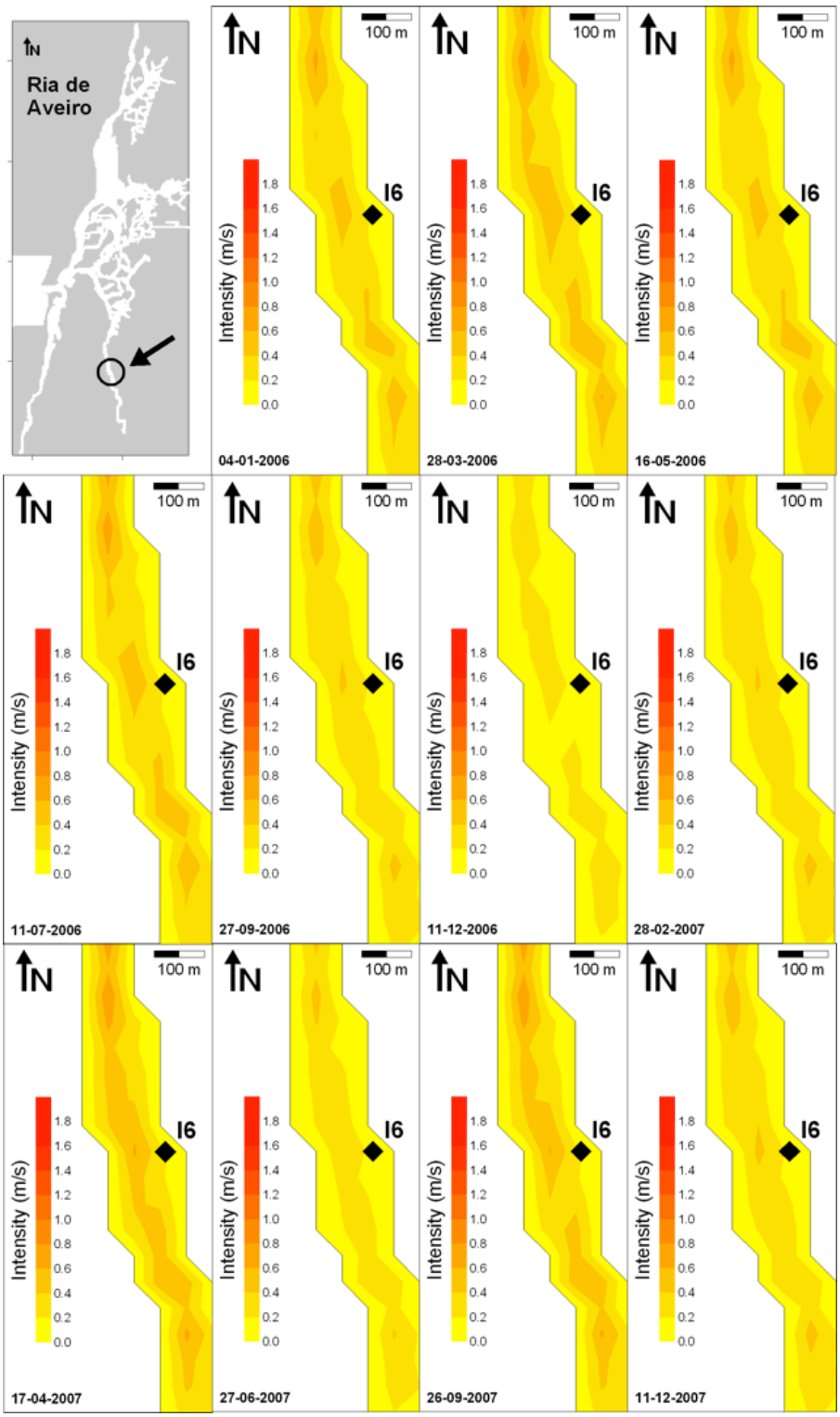

Figure 3.3. Root-mean square velocity field determined from instantaneous numerically predicted velocity values for a complete tidal cycle centered in each sampling period for the hydrological stable station (brackish water zone – I6) of the estuarine system Ria de

3.3.4. TOTAL AND PARTICLE-ATTACHED BACTERIA

In the marine zone (N1), the total bacterial numbers (Figure 3.4; Table 3-III) ranged from 0.5 to 7.6 × 10^9 cells L^{-1} (average 2.5 × 10^9± 2.33 × 10^9 cells L^{-1}) (EF=1.2 ± 0.39). The differences in the total cell abundance between the two compartments were not statistically significant (ANOVA, P>0.05; Table 3-IV).

Cell abundances at the SML and in UW were significantly correlated (P=0.900, N=11) (Table 3-V). The percentage of particle-attached bacteria (Table 3-III) was highly variable and was, on average, two times higher (ANOVA, P<0.005; Table 3-IV) at the SML (20.8 ± 28.98) than that in UW (10.7 ± 8.53). Both total and particle-attached bacteria correlated with wind speed at SML and UW (Table 3-V). The EF of the total bacterial abundance was negatively correlated with the wind intensity (P=−0.682, N=11). In the brackish water zone (I6), the total bacterial numbers varied between 1.6 and 20.5 × 10^9 cells L^{-1} (average 5.2 × 10^9± 4.22 × 10^9 cells L^{-1}) (Figure 3.4; Table 3-III) and the differences in abundance between SML and UW were not significant (ANOVA, P>0.05; Table 3-IV). The percentage of particle-attached bacteria (Table 3-III) was three times higher (anova, P<0.05) at the SML (41.8 ± 28.98) than that in UW (17.3 ± 11.64). The total bacterial numbers in the SML and UW were significantly correlated (P=0.809; Table 3-V), but the level of correlation was not significant for the fraction of particle-attached bacteria. Both total and particle-attached bacteria in UW correlated with wind speed (Table 3-V).

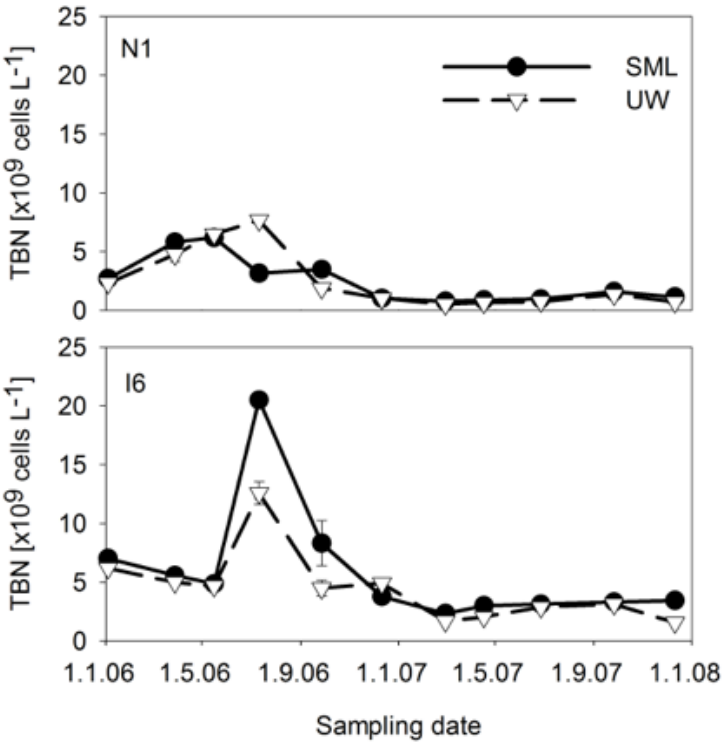

Figure 3.4. Profile of variation of the total bacterial number (TBN) in the SML and UW at two hydrodynamic distinct zones (marine zone –N1 and brackish water zone – I6) in the estuarine system Ria de Aveiro during the sampling period. Errors bars represent the SD of three replicates.

Table 3-III. Total bacterial number (TBN), particle-attached bacteria (PAB) and bacterial biomass productivity (BBP) at the surface microlayer (SML) and in underlaying water (UW) in the marine (N1) and brackish water (I6) zones of the estuary Ria de Aveiro. (Average ± standard deviation); EF – Enrichment factor;

TBN (x10^9 cells L^{-1})			PAB (x10^9 cells L^{-1})			% PAB		PBB (µg C L^{-1} h^{-1})		
SML	UW	EF	SML	UW	EF	SML	UW	SML	UW	EF
Marine zone [N1]										
2.5±1.97 (n=11)	2.5±2.55 (n=11)	1.2±0.39	0.7±0.98 (n=11)	0.3±0.41 (n=11)	3.8±4.95	20.8±28.98	10.7±8.53	2.9±4.62 (n=11)	8.2±8.84 (n=11)	0.3±0.31
Brackish water zone [I6]										
5.9±5.17 (n=11)	4.5±3.10 (n=11)	1.3±0.42	3.3±5.62 (n=11)	0.7±0.39 (n=11)	5.9±8.06	41.8±28.98	17.3±11.64	18.5±23.4 (n=11)	7.9±5.15 (n=11)	3.0±3.37

Table 3-IV. Results of the One-Way ANOVA test to the differences between the SML and UW samples for microbial parameters in the marine (N1) and Brackish water (I6) zones of the estuarine system Ria de Aveiro.TBN- Total Bacterial Number; PAB – Particle-attached bacteria; BBP – Bacterial Biomass Productivity

	TBN	PAB	BBP
Marine zone [N1]	p = 0.452 (N = 64)	p = 0.025 (N = 60)	p = 0.003 (N = 64)
Brackish water zone [I6]	p = 0.070 (N = 64)	p = 0.000 (N = 64)	p = 0.013 (N = 65)

p - significance level;

Table 3-V. Spearman correlation between Surface microlayer and Underlying waters (SML/UW) and between the different microbiological parameters and abiotic factors in the marine (N1) and brackish water (I6) zones of the estuarine system Ria de Aveiro. TBN- Total Bacterial Number; PAB – particles-attached bacteria; BBP - Bacterial Biomass Productivity; EF – Enrichment factor;

	TBN	PAB	BBP
Marine zone [N1]			
SML/UW	p = 0.900 (**) (N=11)	p = 0.903(**) (N=11)	p = 0.664(*) (N=11)
SML	Wind – p = 0.855(**) (N=11)	Wind - p=0.782(**) (N=11)	N.S.
UW	Wind - p=0.891(**) (N=11)	Wind – p=0.855(**) (N=11)	N.S.
EF	Wind – p = -0.682(*) (N=11)	N.S.	N.S.
Brackish water zone [I6]			
SML/UW	p = 0.809 (**) (N=11)	p = 0.327 (N.S) (N=11)	p = 0.055 (N.S.) (N=11)
SML	N.S.	N.S.	N.S.
UW	Wind - p = 0.809 (**) (N=11)	Wind – p = 0.607 (*) (N=11)	N.S.
EF	N.S.	N.S.	N.S.

(**) Correlation is significant at the 0.01 level (2-tailed);
(*) Correlation is significant at the 0.05 level (2-tailed);
N.S. – not significant;

3.3.5. BACTERIAL BIOMASS PRODUCTIVITY (BBP)

At the marine zone, BBP in the SML was significantly lower (EF=0.3 ± 0.31; anova, P<0.05; Table 3-V) than that in UW, ranging from 0.1 to 32.1 µg C L^{-1} h^{-1} (Figure 3.5; Table 3-III). BBP in the SML and in UW were significantly correlated (P=0.664, N=11; Table 3-IV), but significant correlations were not found with other biotic or abiotic variables. In the station I6, BBP was significantly (anova, P<0.05; Table 3-V) enhanced at SML (EF=3.0 ± 3.37), varying between 0.5 and 81.4 µg C L^{-1} h^{-1} (Figure 3.5; Table 3-III). Significant correlations between bacterial activities at the SML and in UW or between biotic or abiotic factors were not observed (Table 3-IV).

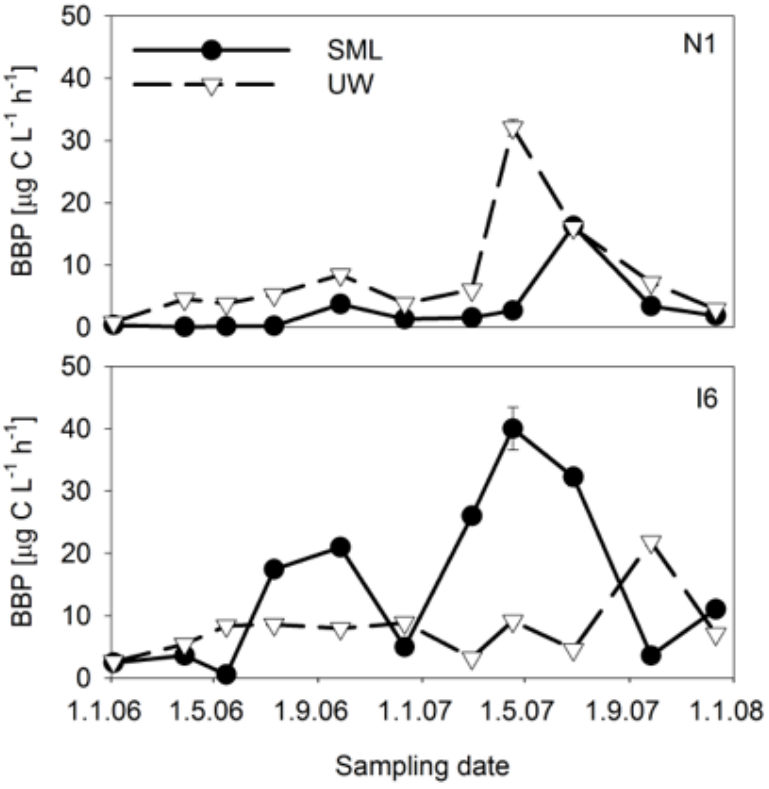

Figure 3.5. Distinct profile of variation of bacterial biomass production (BBP) in the SML and UW in the more hydrodynamic zone (marine zone –N1) and stable zone (brackish water zone – I6) in the estuarine system Ria de Aveiro. Errors bars represent the SD of three replicates.

3.4. DISCUSSION

The abundance and activity of bacteria of the SML of Ria de Aveiro reflect estuarine hydrodynamics in two major ways: (1) in the more hydrodynamic zone (marine zone), BBP was lower than that in UW, whereas in the more stable zone (brackish water zone), it was enhanced and (2) the particle-attached bacteria were enriched in both estuarine zones.

The SML environment is usually described as a highly productive compartment, where frequently high rates of bacterial carbon production (Obernosterer *et al.*, 2005) and mineralization (Obernosterer *et al.*, 2005; Reinthaler *et al.*, 2008) are observed. However, lower bacterial biomass production rates of bacterioneuston when compared with bacterioplankton were also reported in diverse studies (Obernosterer *et al.*, 2008; Stolle *et al.*, 2009).

In the present study, both higher and lower rates of BBP in the SML in comparison with UW were observed, depending on the location in the estuary. In the marine zone, bacterioneuston was on average 10 times less active than bacterioplankton, but in the brackish water zone, bacterial activity in the SML was three times higher than that observed at the UW. These distinct metabolic responses in the two estuarine compartments could not be explained by any of the physical and chemical variables determined, suggesting that bacteria in the estuarine SML environment are controlled by different factors such as hydrodynamics, meteorological conditions or nutrient availability.

The major differences between the two studied zones are their hydrodynamic characteristics and bathymetry (Dias *et al.*, 2000, 2001). Water circulation is one of the main physical processes that affects and controls many ecological processes in an estuary (Day *et al.*, 1989). In Ria de Aveiro, the combination of hydrodynamic and morphologic characteristics determines the spatial distribution of nutrients and primary production (Lopes *et al.*, 2010), the dynamics of suspended particulate matter (Dias *et al.*, 2003) and water residence time (Dias *et al.*, 2001; Dias *et al.*, 2003). It is expected that the SML environment reflect the hydrodynamic aspects of the estuary.

The application in the present work of a previously developed numerical model to the Ria de Aveiro (Dias & Lopes, 2006b, 2006a) showed that in the marine zone, water currents are stronger than that at the brackish water zone. Under the marine influence, intense water currents in the outer estuary zone may produce turbulent mixing, promoting the interchange of bacterial cells between the SML and the UW column. In areas under the action of mixing processes induced by wind, waves, currents, bubbles and tides, the materials in the microlayer and subsurface waters are in continuous interchange and the speed of the renewal process is dependent on the degree of water mixing (Liss *et al.*, 1997). The exchange of bacteria between the two compartments is supported by the significant correlation between total and particle-attached bacteria abundances at the SML and UW. As a consequence of the constant mixing between SML and UW, bacteria may have short residence times at the SML that preclude their adaption and metabolic response to the potential organic enrichment of the air–water interface. Therefore, the low rates of BBP observed at SML of this estuarine area might be a consequence of the vertical mixing forced by the hydrodynamic conditions. Moreover, the activity of neustonic and planktonic bacterial communities was

correlated, supporting the idea of a water column source of bacteria to the SML. A high correlation between bacterial abundance or activity at SML and UW was also observed in previous studies and related to the upward of microorganisms attached to buoyant particles or bubble scavenging (Joux *et al.*, 2006).

Bacterial activity at the SML of the brackish water zone was significantly enriched. In this estuarine area, tidal-induced water currents were weak. Moreover, the water residence time is longer, varying between 1 and 2 weeks (Dias *et al.*, 2001). This hydrodynamic stability might reduce vertical mixing, promoting stratification and increasing the residence time of the bacterial community at the surface and allowing the exploration of a potential nutrient enrichment of the SML. The pattern of variation of both particle-attached bacteria and biomass production at SML was not correlated with UW, indicating an uncoupling between the two communities. Calm conditions can induce a succession of physical and chemical characteristics at SML and the uncoupling of bacterial parameters from those of the water column (Stolle *et al.*, 2010).

Although the total bacterial numbers were similar, the fraction of particle attached was significantly enriched at the SML in both marine and brackish water zones of the estuary. Particle-attached bacteria represent a significant fraction of the total bacterial numbers at the SML, constituting on average 20% and 40% of the total abundance in marine and brackish water zones, respectively. Higher percentages of particle-attached bacteria at the SML when compared with subsurface water were also observed in other aquatic environments (Harvey & Young, 1980; Aller *et al.*, 2005; Obernosterer *et al.*, 2005; Cunliffe & Murrell, 2009). The higher concentration of particle-attached bacteria at the SML might be due to the upward of colonized particles from the water column (Stolle *et al.*, 2010) and/or due to in situ colonization (Cunliffe & Murrell, 2009).

In contrast to the dissolved pool, particulate organic matter (POM) enrichment at SML is a consistent feature (Carlson, 1983; Kuznetsova *et al.*, 2004; Obernosterer *et al.*, 2005; Obernosterer *et al.*, 2008; Stolle *et al.*, 2009). This could be related to the aggregation of dissolved and/or particulate matter in the water column and flotation towards the surface. The attachment of bacteria to particles in the planktonic environment is controlled essentially by the number (Iriberri *et al.*, 1987; Unanue *et al.*, 1992) and quality of particles (Simon *et al.*, 2002). Therefore, it is expected that enrichment of particle-attached bacteria at SML is due to a higher number of available particles and/or due to the improvement of their quality. Because of limitations of the amounts of SML water available for analysis, the concentration of POM was not assessed in this study, but other works conducted in Ria de Aveiro showed that suspended matter and POM can be five times more concentrated in SML samples than in UW (C. Prata, unpublished data). Moreover, during the examination of estuarine samples by epifluorescence microscopy for the enumeration of bacterial cells, more particles were observed in the SML samples (data not shown).

The dynamics of particle-attached bacteria at SML and UW was influenced by wind. In both the zones studied, the number of particle-attached bacteria in the water column increased with the wind intensity. Although two orders of magnitude lower than tidal currents, wind induces a residual circulation in Ria de Aveiro, influencing the transport of suspended sediment particles and increasing the turbidity of the estuary, especially in the shallow areas (Dias *et al.*, 2003). An increased number of particles in suspension produces an increment of particle-attached bacteria in the water column of the estuary (Almeida & Alcântara, 1992). In the more hydrodynamic zone of the estuary, the vertical mixing might promote the migration of bacterial colonized particles to the surface, explaining the observed relation between wind and particle-attached bacteria at marine SML. In this particular estuarine zone, we also observed a decrease in the bacterial abundance EF as the wind intensity increased. Wind increases surface pressure and convergent forces (Wheeler, 1975), forcing the collapse of SML and causing the transport of materials downward to the water column.

The present study demonstrates that in a highly dynamic system, such as Ria de Aveiro, bacterial communities may not develop significant adaptations to the peculiar SML environment, but under favorable conditions, they readily increase their activity, with the potential to influence the food webs at the air–water interface. The SML estuarine environment favors the 'attached' way of life of SML communities (Cunliffe & Murrell, 2009), reinforcing the idea of an ecological role based on the detrital food web (Obernosterer *et al.*, 2008). However, the factors (number or/and nature of the particles) that promote the attachment of bacteria to particles in the SML environment, as well as the mechanisms by which hydrodynamic and meteorological variables promote mixing with the water column, influencing the dynamic and activity of bacterial communities, remain to be elucidated. In order to answer these relevant questions, laboratory experiments will be conducted in the future and the individual and combined effects will be simulated.

3.5. CONCLUSION

This work contributes toward a better understanding of the ecology of microbial communities at SML in tidal ecosystems. Estuarine hydrodynamics influences the ecological processes and, therefore, the SML environment. The hydrodynamic characteristics of the estuary Ria de Aveiro influence the properties of the water column and, consequently, the dynamics of bacterial communities at SML. In the marine zone, intense water currents promote a strong mixing of water, circumventing the establishment of distinct SML bacterial communities. In contrast, in the brackish water zone, local hydrodynamic characteristics provide the necessary conditions for the proliferation of active bacterial communities at SML. Estuarine dynamics significantly influence

microbial activity at the air–water interface, explaining differences in bacterial activity and density among the diverse sites in the same system and, possibly, among different systems.

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CHAPTER 4

4. HETEROTROPHIC ACTIVITIES OF NEUSTONIC AND PLANKTONIC BACTERIAL COMMUNITIES IN AN ESTUARINE ENVIRONMENT (RIA DE AVEIRO)

Abstract

The uppermost millimeter of the water column, the surface microlayer (SML), hosts bacterial communities (bacterioneuston) with potential metabolic adaptations to this unique physical and chemical environment. Hydrolysis and monomer incorporation by bacterioneuston and bacterioplankton communities in the estuarine system Ria de Aveiro was investigated and compared during a two-year survey. The study was conducted in two contrasting sites, typifying the marine and brackish water characteristics of the estuary and having different prevailing sources, amounts and composition of organic matter. In the marine zone, bacterioneuston exhibited higher rates of hydrolysis and lower rates of monomer incorporation than bacterioplankton, whereas in the brackish water zone, neustonic and planktonic microbial communities showed similar activities rates. This pattern might result from the different degrees of surface organic and inorganic matter enrichments, which reflect site-specific characteristics, such as hydrodynamics and sources of organic matter composition. In general, estuarine SML environment favors polymer hydrolysis, but inhibits monomer utilization, in comparison with the sub-surface water layers. However, the differences between the two communities tend to attenuate as autotrophic and heterotrophic activities increase in the brackish-water area.

Keywords: Surface microlayer; bacterioneuston; bacterioplankton; bacterial monomer incorporation; bacterial ectoenzymatic activity; estuary; Ria de Aveiro;

4.1. INTRODUCTION

Located at the air-water interface of potentially all aquatic systems, the surface microlayers (SML) are unique microbial habitats, where neustonic microorganisms interact with the atmosphere and the hydrosphere (Cunliffe *et al.*, 2011). The SML comprises the top 1000 µm of the water column (Liss & Duce, 1997) and is physical and chemically distinct from the underlying water (UW) (Zhang *et al.*, 2003). Diverse physical processes such as diffusion, turbulent mixing, scavenging and transport by bubbles and buoyant particles (Liss *et al.*, 1997) contribute to the concentration of numerous organic and inorganic compounds at the SML (Henrichs & Williams, 1985) (Sieburth *et al.*, 1976; Williams *et al.*, 1986; Hunter, 1997; Liss & Duce, 1997), most of which originates in the water column (Liss & Duce, 1997; Kuznetsova *et al.*, 2004). Increasing evidences support the concept that SML is a gelatinous biofilm (Sieburth, 1983; Wurl & Holmes, 2008; Cunliffe & Murrell, 2009), where colloidal material (Bigg *et al.*, 2004) and transparent exopolymer particles (TEP) are enriched (Kuznetsova *et al.*, 2005; Cunliffe *et al.*, 2009; Wurl *et al.*, 2009).

The organic enrichment of the SML likely favors heterotrophic activity (Obernosterer *et al.*, 2005), fueling highly active communities of microorganisms (Sieburth *et al.*, 1976). Microbial dissolved organic matter (DOM) transformation processes such as enzymatic hydrolysis (Munster *et al.*, 1998; Kuznetsova & Lee, 2001; Mudryk & Skórczewski, 2004) and uptake (Carlucci *et al.*, 1991; Garabétian, 1991; Munster *et al.*, 1998; Santos *et al.*, 2009) are enhanced at the SML compared with in the water column likely due to the greater concentrations of DOM at this interface (Kuznetsova & Lee, 2001). Despite of a potentially higher availability of substrates at the SML, bacterioneuston growth efficiencies were found to be very low, suggesting low accessibility of substrates for bacteria at this interface (Reinthaler *et al.*, 2008). Therefore, the activity of bacteria at the SML might reflect chemical and physical processes that contribute to the organic enrichment at the air-water interface and to organic matter accessibility for bacterial utilization.

Organic matter in estuaries derive from multiple natural and anthropogenic allochthonous and autochthonous sources produced along a freshwater to a seawater continuum (Bianchi, 2006). Characteristic gradients of organic carbon and inorganic nutrients in estuaries support high rates of primary (Boyer *et al.*, 1993; Sorokin & Sorokin, 1996; Gaulke *et al.*, 2010) and secondary production (Goosen *et al.*, 1999; Ducklow, 2002). Additionally to the multiplicity of organic sources, physical estuarine inherent characteristics such as wind (Stolle *et al.*, 2010) and water circulation (Santos *et al.*, 2011) change the dynamic of SML, influencing the distribution and activity of microorganisms at the SML and in the water column and, therefore, the study of organic matter transformation in these ecosystems are challenging. This work aimed to identify patterns of variation and primary environmental regulators of heterotrophic activities of neustonic and

planktonic communities in an estuarine system. In order to achieve that goal, two contrasting zones of the estuary Ria de Aveiro, with different amounts and prevailing sources of organic matter and differently impacted by estuarine hydrodynamic characteristics were chosen.

4.2. MATERIALS AND METHODS

4.2.1. STUDY SITE

Ria de Aveiro (40º 38'N, 8º 45'W; Figure 4.1) is a shallow tidal lagoon situated on the Northwest Atlantic coast of Portugal, separated from the sea by a sand barrier. The lagoon covers an area of 66 and 83 km^2 at low and high tide, respectively. It exchanges with the sea a volume of water of 137 Mm^3 for maximum spring tide and 35 Mm^3 for minimum neap tide (Dias *et al.*, 2000). Several rivers carry fresh water into the lagoon with an average water input of 1.8 Mm^3 during a tidal cycle (Dias *et al.*, 2003). The lagoon has a complex topography, with four main channels (Espinheiro, S. Jacinto, Mira and Ílhavo) spreading from the mouth in diverse streams, forming a complex estuarine system (Dias *et al.*, 2001). For this study two sampling stations were selected: the station N1, located at the entrance of the estuary, represents the marine conditions, and the stations I6, located at the Ílhavo channel, represents the brackish water conditions of estuary.

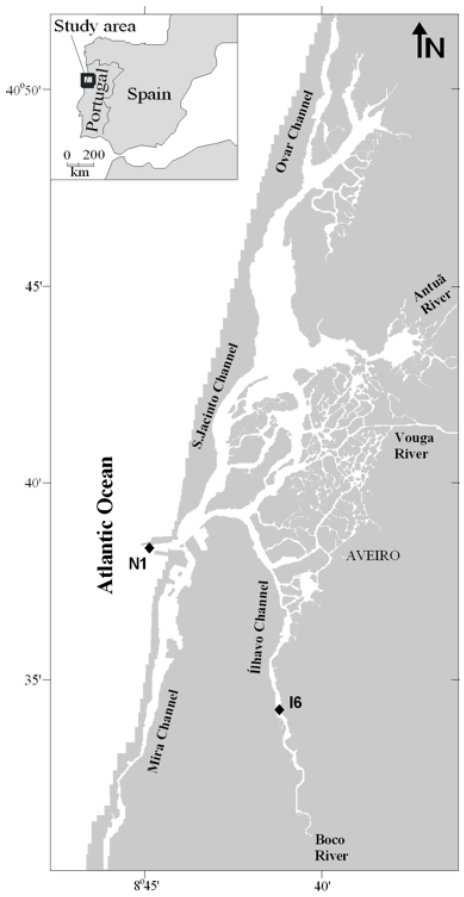

Figure 4.1. The estuarine system Ria de Aveiro with the indication of sampling stations. Station N1, located at the Canal de Navegação typifies the marine zone of the estuary and the station I6, located at the Ílhavo channel, the brackish water zone of the estuary.

4.2.2. SAMPLING

Sampling was carried out at low tide, approximately every two months during 2006 and 2007. SML samples were collected with a Plexiglas plate (Harvey & Burzell, 1972), which collects roughly the upper 60–100 µm water layer. The plate dimensions were 0.25 m wide x 0.35 m long and 4 mm thick. Before sampling, the plate was cleaned with ethanol, rinsed with sterile distilled water and finally with water from the sampling site. The water adhering to the plate during immersion was removed from both sides forcing the plate between two Teflon sheets and collecting the water into a sterilized glass bottle. Samples from UW were collected with a horizontal Van Dorn bottle at 20 cm depth and transferred to a sterilized glass bottle. Samples were kept cold

during the transport to the laboratory and processed within 2-3 hours of collection.

4.2.3. ENVIRONMENTAL PARAMETERS

Temperature and salinity were measured in the field using a WTW LF 196 Conductivity Meter (Wissenschaftlich Technische Werkstätten, Germany), and dissolved oxygen concentration was measured with a WTW OXI 320 oxygen meter (WissenschaftlichTechnische Werkstätten, Germany). Temperature and dissolved oxygen concentration were only determined for UW, because the collection of the SML takes a considerable time during which changes in temperature and oxygen concentration are likely to occur.

Chlorophyll a (Chl a) concentration was estimated fluorimetrically (Yentsch & Menzel, 1963) after filtration of 0.5 L triplicate subsamples through Whatman GF/F filters and overnight cold extraction in 90% (v/v) acetone. Suspended particulate matter (SPM) concentration was determined after filtration of triplicate 0.5-L water aliquots through pre-weighted and pre-combusted Whatman GF/F filters. The filters were dried at 60 ºC for 24 h, and SPM was calculated as the increase in dry weight. Particulate organic matter (POM) was determined from the further decrease in weight after 4 h incineration at 550°C (Parsons *et al.*, 1989). For nutrient analysis, water subsamples were filtered through MSI acetate membranes with 0.45µm pore size and stored at -20 °C in acid-cleaned polyethylene flasks until determination. Orthophosphate and nitrite were quantified using methods described in (Hansen & Koroleff, 2007). Nitrate was assayed using an adaptation of the spongy cadmium reduction technique (Jones, 1984), with the nitrite value subtracted from the total.

4.2.4. METEOROLOGICAL PARAMETERS

Wind speed data coincident with the period of sampling were recorded at the meteorological station of the University of Aveiro, located close to the sampling area (http://climetua.fis.ua.pt/).

4.2.5. MICROBIOLOGICAL PARAMETERS

The number of total and particle-attached bacteria was determined by epifluorescence microscopy using a Leica “DMLS” microscope equipped with a I 2/3 filter for blue light. Particle-attached cells were counted directly and distinguished from free-living cells on the same slide. Three replicates for each sample were filtered through 0.2 µm black polycarbonate membranes (GE

Osmonics) and stained with 0.03% acridine orange (Hobbie *et al.*, 1977). At least 200 cells or 20 microscope fields were counted for each replicate measurement.

The heterotrophic metabolism of the ^{14}C-labeled monomer acetate (SA 2.04 GBq $mmol^{-1}$, 55.0 mCi $mmol^{-1}$), glucose (SA 11.5 GBq $mmol^{-1}$, 310 mCi $mmol^{-1}$) and leucine (SA 11.3 GBq $mmol^{-1}$, 306 mCi $mmol^{-1}$) was described by the parameter Vm (maximum uptake velocity) following the procedure described by Gocke (1977). A final saturation concentration of 430 nM of each radioactively labeled substrate was added to 10 mL aliquots of the water samples. Substrate concentrations were chosen after kinetic analysis. Incubations were carried out for 2 h at *in situ* temperature. Cells were collected on 0.2-µm pore-size filters (GE Osmonics) and radioactivity was read in a liquid scintillation counter (LS 6000 IC, Beckman,) using UniverSol (ICN Biomedicals) as scintillation cocktail. Radioactive labeled acetate, glucose and leucine were obtained from Amersham.

Extracellular enzymatic activity was determined fluorimetrically (Jasco FP-777 fluorometer) as the maximum hydrolysis rate (Hm) of model substrates (Hoppe, 1991). The following substrates were used for the indicated enzyme (substrate for enzyme): L-leucine-7-amido-4-methyl-coumarin hydrochloride (Fluka) for leucine aminopetidase (E.C. 3.4.11.1) (Kanaoka *et al.*, 1977); 4-methylumbelliferyl-β-glucopyranoside (Fluka) for β-glucosidase (E.C. 3.2.1.21) (Daniels *et al.*, 1981); 4-methylumbelliferyl-β-D-galactopyranoside (Sigma) for β-galactosidase (EC 3.2.1.23) (Vernet *et al.*, 1993). 4-methylumbelliferyl-fosfate (Sigma) for alkaline phosphatase (E.C. 3.1.3.1) (Roberts *et al.*, 1991); 4-methylumbelliferyl-acetate (Sigma) for esterase (E.C. 3.1.1.2); 4-methylumbelliferyl-palmitate (Sigma) for lipase (E.C. 3.1.1.3) (Gajewski *et al.*, 1997). The substrates were added at saturating concentrations (10 mM of acetate, 4-methylumbelliferyl-β-glucopyranoside, L-leucine-7-amido-4-methyl-coumarin hydrochloride and 4 - methylumbelliferyl-fosfate; 4 mM of 4-methylumbelliferyl-palmitate and 4 - methylumbelliferyl - β - D - galactopyranoside). Wavelengths for excitation and emission were 380 to 440 nm for MCA (7-amino-4-methylcoumarine) and 360 to 450 nm for MUF (4-methylumbelliferone). Measurements were made in 3 replicates for each sample after 2h, for MCA, and 18 h for MUF. Incubations were made at *in situ* temperature. Calibration was performed by adding a series of 6 to 8 concentrations of the fluorescent products (0 to 500 nM for MUF and 0 to 6 µM for MCA) to a pool of water from the 2 sampling stations.

4.2.6. DATA ANALYSIS

Microbiological parameters determined for SML and UW were compared and expressed as the enrichment factor (EF) defined as $EF = [X]_{SML}/[X]_{UW}$, where [X] is the concentration of a given

parameter (GESAMP, 1995). The statistical analysis of microbiological data was performed with the SPSS 15.0 (SPSS Statistics) software. The relations between the different parameters were examined using a Spearman correlation. One-Way ANOVA was used to determine the significance of the differences observed in microbial parameters between the two layers. Normal distribution was assessed by the Kolmogorov-Smirnov test and the homogeneity of variances by the Levene test. A value of $p<0.05$ was considered significant.

4.3.RESULTS

4.3.1.PHYSICAL AND CHEMICAL PARAMETERS

The values of the physical and chemical parameters determined in the SML and UW are shown in Table 4-I. Salinity was similar in the SML and UW, ranging from 10.1 to 36.6 psu (average 28.8 ± 8.56 psu) at station N1 and from 0.2 to 36.8 psu (average 20.0 ± 12.01 psu) at station I6. Temperature varied between 10.3 and 24.4 ºC, showing a typical seasonal profile of variation. The average temperature was 16.0 ± 2.56 ºC at station N1 and 17.5 ± 5.21 ºC at station I6. Oxygen concentration ranged from 1.9 to 12.3 mg L^{-1} (average 7.2 ± 3.3 mg L^{-1}) and the seasonal profile of variation was similar at both zones of the estuarine system.

4.3.2.METEOROLOGICAL PARAMETERS

The average daily wind speed (Table 4-I) during the sampling, varied between 1.0 and 40.3 ms^{-1} (average 13.1±12.4 ms^{-1}), and was 8 times more intense during 2006 compared with 2007.

4.3.3.CHARACTERISTICS OF THE UNDERLYING WATER (UW)

The values of the concentration of chlorophyll a, suspended and particulate matter, and nutrients determined in water column are shown in Table 4-II. Chlorophyll a concentration followed a typical seasonal profile of variation, ranging from 0.7 to 5.8 µg L^{-1} (average 2.9 ± 1.6 µg L^{-1}) at the marine zone and from 1.3 to 9.9 µg L^{-1} (average 4.9 ± 2.7 µg L^{-1}) at the brackish water zone. Suspended particulate matter (SPM) concentration varied between 26.1 and 74.0 mg L^{-1} (average 56.4 ± 15.0 mg L^{-1}), and between 30.0 and 79.1 mg L^{-1} (average 53.6 ± 17.9 mg L^{-1}) at marine and brackish water zones, respectively. The concentration of particulate organic matter (POM) was on average, 13.8±4.8 mg L^{-1} (range 7.2 to 21.5 mg L^{-1}) at the marine zone and 12.4±5.81 mg L^{-1} (range 5.3 to 23.9 mg L^{-1}) at the brackish water zone. The concentration of nitrates plus nitrites (NO_3^- + NO_2^-) ranged from 3.5 to 19.4 µM (average 7.4 ± 5.0 µM) at the marine zone and from 6.3 to 84.0 µM (average 29.1 ± 23.7 µM) at the brackish water zone.

Table 4-I. Physical and chemical determined in the surface microlayer (SML) and underlying water (UW) of the marine [N1] and brackish water [I6] zones of the estuarine system Ria de Aveiro, and wind speed during the sampling events.

Year	2006						2007				
Month	Jan	Mar	May	Jul	Sep	Dec	Feb	Apr	Jun	Sep	Dec
Marine zone [N1]											
Salinity [psu]											
SML	34.0	23.6	34.9	36.5	35.9	11.0	23.3	34.4	33.1	36.6	17.3
UW	32.9	23.3	34.2	36.3	35.5	10.1	23.2	33.9	33.2	33.2	17.2
Temperature [ºC]											
UW	12.7	15.0	18.2	18.2	19.4	13.2	14.0	19.0	17.4	16.0	13.0
Oxygen [mg L^{-1}]											
UW	10.5	9.1	5.3	ND	2.7	2.5	9.7	10.9	ND	8.3	11.3
Brackish water zone [I6]											
Salinity [psu]											
SML	20.8	7.9	27.3	36.8	30.0	0.3	3.8	23.5	19.6	35.8	14.6
UW	20.7	7.4	26.9	36.5	30.3	0.2	3.7	23.1	19.5	35.9	14.5
Temperature [ºC]											
UW	11.0	16.7	24.1	24.4	22.1	10.3	13.5	20.6	19.8	18.2	11.8
Oxygen [mg L^{-1}]											
UW	12.3	5.9	3.4	ND	1.9	2.2	7.9	7.9	ND	6.5	9.7
Wind intensity [m s^{-1}]	11.4	40.3	27.3	18.4	17.0	15.7	1.0	2.0	4.9	4.0	1.6

N. D. – Not determined.

Phosphate concentration at the marine zone of the estuary varied between 0.2 and 16.2 μM (average 2.6 ± 4.5 μM) and, at the brackish water zone between 0.4 and 41.0 μM (average 5.7 ± 11.8 μM).

4.3.4. TOTAL AND PARTICLE-ATTACHED BACTERIAL NUMBERS

Total bacterial numbers (TBN) in the SML and UW were similar (ANOVA $p>0.05$) both at marine (average EF=1.2) and brackish water (average EF=1.3) zones, varying between 0.5 and 20.5 x 10^9 cells L^{-1} (Table 4-III). Nevertheless, the numbers of particle-attached bacteria (PAB) were significantly enriched in the SML (average EF=4.6; ANOVA, $p<0.05$) of both estuarine zones, ranging from 0.03 to 19.7 x 10^9 cells L^{-1}. The fraction of bacteria attached to particles was enriched in the SML (2 to 3 times), representing on average, 15.5 % at station N1 and 29.5 % at station I6.

Table 4-II. Concentration of chlorophyll a (Chl a), suspended particulate matter (SPM), particulate organic matter (POM), nitrate + nitrite (NO_3^- + NO_2^-) and phosphate (PO_4^{3-}) in the underlying water [UW] of the marine [N1] and brackish water [I6] zones of the estuarine system Ria de Aveiro.

Year	2006						2007				
Month	Jan	Mar	May	Jul	Sep	Dec	Feb	Apr	Jun	Sep	Dec
Marine zone [N1]											
Chl a [µg L^{-1}]	2.4±0.12	2.0±0.12	4.2±0.07	5.8±0.07	1.8±0.01	1.8±0.05	3.8±0.19	4.9±0.13	3.3±0.09	1.4±0.16	0.7±0.07
SPM [mg L^{-1}]	46.6±0.96	41.5±1.89	67.1±1.14	74.0±0.96	65.3±1.30	26.1±2.18	43.7±0.90	58.2±1.31	73.3±1.63	61.3±0.93	63.3±0.75
POM [mg L^{-1}]	11.4±0.50	10.5±0.43	16.7±0.33	21.5±0.33	18.2±0.38	7.2±0.81	12.5±0.66	11.7±0.85	21.2±0.96	10.7±0.82	10.5±0.49
NO_3^- + NO_2^- [µM]	11.3	7.6	3.6	4.9	5.9	19.4	3.5	5.6	3.6	12.3	3.5
PO_4^{3-} [µM]	1.0±0.25	1.0±0.01	1.0±0.01	0.8±0.01	1.7±0.10	16.2±0.04	1.3±0.01	0.2±0.11	1.2±0.06	1.9±0.06	2.7±0.04
Brackish water zone [I6]											
Chl a [µg L^{-1}]	3.0±0.13	4.8±0.12	9.9±0.16	7.6±0.05	3.5±0.01	3.4±0.25	6.5±0.02	8.2±0.15	3.9±0.02	2.2±0.07	1.3±0.06
SPM [mg L^{-1}]	32.3±0.96	35.9±0.25	70.5±1.98	76.5±1.52	57.7±1.32	30.0±0.50	48.9±0.81	45.2±2.22	79.1±2.97	68.9±3.71	44.4±1.64
POM [mg L^{-1}]	8.7±0.57	7.3±0.43	14.6±0.34	20.0±0.34	16.9±0.86	5.3±0.52	10.0±0.25	9.4±0.19	23.9±2.75	11.9±0.75	8.3±0.16
NO_3^- + NO_2^- [µM]	39.9	60.0	18.3	18.2	24.2	14.8	84.0	23.8	6.6	24.6	6.3
PO_4^{3-} [µM]	1.3±0.25	1.4±0.12	2.0±0.01	2.5±0.12	4.4±0.20	41.0±1.45	3.1±0.01	0.4±0.04	1.5±0.04	3.6±0.25	1.1±0.01

Table 4-III. Total bacterial number (TBN), particles-attached bacteria (PAB) and the fraction of particles-attached bacteria (%PAB) at the surface microlayer (SML) and underlying water (UW) in the marine (N1) and brackish water (I6) zones of the estuarine system Ria de Aveiro. (Average ± Standard deviation); EF – Enrichment factor;

	TBN (x10^9 cells L^{-1})			PAB (x10^9 cells L^{-1})			% PAB	
	SML	UW	EF	SML	UW	EF	SML	UW
Marine zone [N1]	2.5±1.97 (n=11)	2.5±2.55 (n=11)	1.2±0.39 (N.S)	0.7±0.98 (n=11)	0.3±0.41 (n=11)	3.8±4.95 (*)	20.8±28.98 (n=11)	10.7±8.53 (n=11)
Brackish water zone [I6]	5.9±5.17 (n=11)	4.5±3.10 (n=11)	1.3±0.42 (N.S)	3.3±5.62 (n=11)	0.7±0.39 (n=11)	5.9±8.06 (**)	41.8±28.98 (n=11)	17.3±11.64 (n=11)

(**) ANOVA $p<0.01$;
(*) ANOVA $p<0.05$;
N.S – not significant (ANOVA $p>0.05$);

4.3.5. BACTERIAL HETEROTROPHIC ACTIVITIES

4.3.5.1. INCORPORATION OF MONOMER

The variation profiles of the maximum rates of monomer incorporation (Vm) are shown in the Figure 4.2. The highest Vm values of leucine (30.9 nmol L^{-1} h^{-1}), glucose (84.0 nmol L^{-1} h^{-1}) and acetate (465.6 nmol L^{-1} h^{-1}) incorporation were observed at the brackish water zone of the estuary. At the marine zone, Vm of leucine (average EF = 0.2), glucose (average EF = 0.3) and acetate (average EF = 0.3) incorporation by bacterioneuston was always significantly lower (ANOVA, $p<0.05$) than in bacterioplankton (Table 4-IV). At the brackish water zone, leucine (average EF = 1.0), glucose (average EF = 0.8) and acetate (average EF = 1.1) incorporation Vm

values in the SML were similar to those observed in the UW (ANOVA, p>0.05) (Table 4-IV)

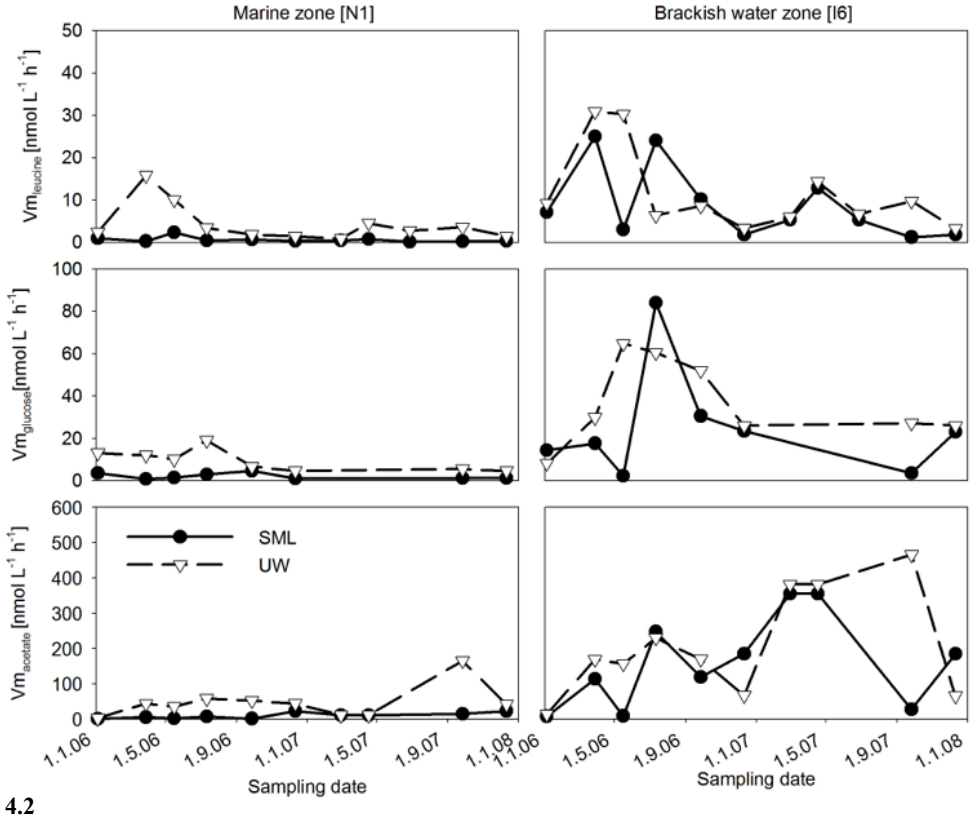

Figure 4.2
Bacterial incorporation rate (Vm) of leucine, glucose and acetate in the surface microlayer (SML) and underlying water (UW) at the marine (N1) and brackish water (I6) zones of the estuarine system Ria de Aveiro during the sampling period.
Error bars represent the standard deviation of three replicates..

4.3.5.2. RATES OF POLYMERS HYDROLYSIS

4.3.5.2.1. AMINOPEPTIDASE ACTIVITY

The enzyme aminopeptidase (Leu-AMPase) showed the highest rates of activity at both studied sites of the estuarine system (Table 4-IV; Figure 4.3). Hm of this enzyme varied between 420.1 and 6489.2 nmol L^{-1} h^{-1} and the highest values were observed at the brackish water zone. The activity of Leu-AMPase was similar (ANOVA p>0.05) and correlated in the SML and UW at both marine (average EF =1.0) and brackish water (average EF=1.3) zones.

Table 4-IV. Bacterial incorporation rate (Vm) of leucine, glucose and acetate and hydrolyze rate (Hm) of aminopeptidase (Leu-AMPase), β-glucosidase (β-GlCase), ß-galactosidase (ß-GalCase), alkaline phosphatase (APase), esterase and lipase in the surface microlayer (SML) and underlying water (UW) at the marine (N1) and brackish water (I6) zones of the estuarine system Ria de Aveiro. (Average ± Standard deviation); EF – Enrichment factor;

	Marine zone [N1]			Brackish water zone [I6]		
	SML	UW	EF	SML	UW	EF
			Vm			
Leucine	0.6±0.62 (n=11)	4.4±5.55 (n=11)	0.2±0.16 (**)	8.9±8.51 (n=11)	11.7±9.83 (n=11)	1.0±0.99 (N.S)
Glucose	2.2±1.46 (n=8)	9.5±5.10 (n=8)	0.3±0.19 (**)	24.9±25.80 (n=8)	36.8±19.85 (n=8)	0.8±0.59 (N.S)
Acetate	9.5±8.33 (n=10)	53.8±45.36 (n=10)	0.3±0.31 (**)	162.1±132.90 (n=10)	206.8±149.20 (n=10)	1.1±0.97 (N.S)
			Hm			
Leu-AMPase	1708±2253.1 (n=8)	1402±1320.5 (n=9)	1.0±0.29 (N.S)	2948±1319.2 (n=10)	2473±1628.8 (n=10)	1.3±0.51 (N.S)
β-GlCase	307.2±381.21 (n=7)	133.1±212.96 (n=8)	7.3±12.54 (**)	450.2±636.05 (n=9)	327.8±586.61 (n=9)	1.3±0.33 (N.S)
ß-GalCase	18.9±12.12 (n=10)	11.6±9.40 (n=10)	3.6±6.18 (**)	103.7±109.82 (n=10)	41.4±39.17 (n=10)	2.6±1.96 (**)
APase	239.1±218.72 (n=9)	86.7±103.55 (n=9)	6.6±10.91 (**)	170.4±127.72 (n=9)	121.4±106.72 (n=9)	2.1±2.05 (*)
Esterase	532.2±462.67 (n=8)	535.2±554.00 (n=9)	1.1±0.66 (N.S)	628.2±561.50 (n=9)	457.9±423.36 (n=9)	1.5±0.99 (N.S)
Lipase	4.9±2.69 (n=8)	2.7±1.14 (n=8)	2.0±1.41 (**)	3.8±2.13 (n=8)	2.9±1.77 (n=8)	1.5±0.91 (n.s)

(**) ANOVA $p<0.01$;
(*) ANOVA $p<0.05$;
N.S – not significant (ANOVA $p>0.05$);

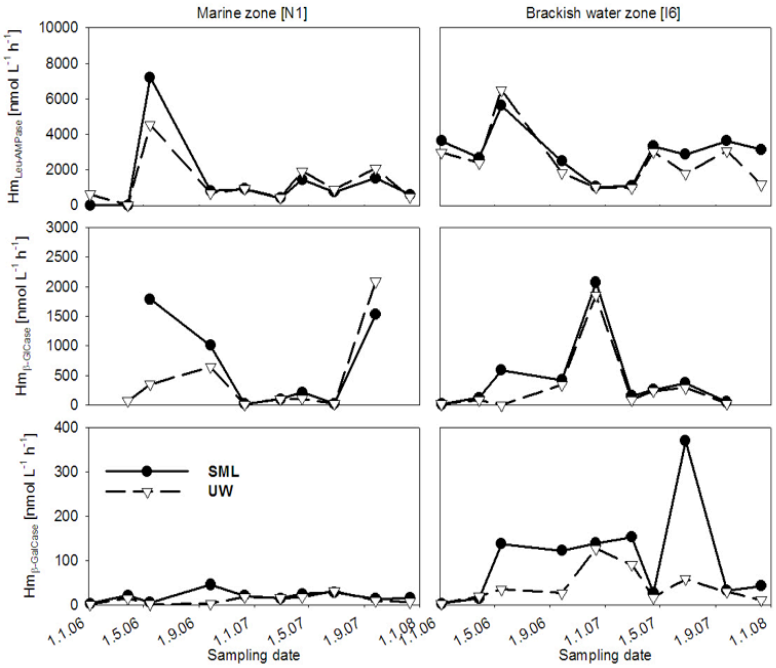

Figure 4.3
Hydrolysis rates (Hm) of aminopeptidase (Leu-AMPase), ß-glucosidase (ß-GlCase) and ß-galactosidase (ß-GalCase) in the surface microlayer (SML) and underlying water (UW) at the marine (N1) and brackish water (I6) zones of the estuarine system Ria de Aveiro during the sampling period.
Error bars represent the standard deviation of three replicates.

4.3.5.2.2. ß-GLUCOSIDASE AND ß-GALACTOSIDASE ACTIVITIES

β-glucosidase (β-GlCase) and ß-galactosidase (ß-GalCase) Hm ranged from 3.6 to 2071.0 nmol L^{-1} h^{-1} and from 1.1 to 369.0 nmol L^{-1} h^{-1}, respectively (Table 4-IV; Figure 4.3). The highest Hm values of both enzymes were observed at the brackish water zone. β-GlCase potential activity was, on average, 7 times higher (ANOVA $p<0.05$) in the SML compared with UW at the marine zone and similar (average EF = 1.3; ANOVA $p>0.05$) at the brackish water zone. ß-GalCase Hm was, on average, 3.6 and 2.6 times higher (ANOVA $p<0.05$) in the SML than in the UW at the marine and brackish water zones, respectively.

4.3.5.2.3. PHOSPHATASE ACTIVITY

Alkaline phosphatase (APase) Hm ranged from 7.2 to 727.4 nmol L^{-1} h^{-1} and the highest value was observed in the SML of the marine zone (Table 4-IV; Figure 4.4). Here, the activity of this enzyme was, on average, 6.6 times higher (ANOVA $p<0.05$) in the SML than in the UW. At the brackish water zone, APase Hm was also significantly higher in the SML environment (average EF=2.1; ANOVA $p<0.05$).

4.3.5.2.4.ESTERASE

Esterase Hm varied between 2.6 and 1840.1 nmol L^{-1} h^{-1} and the highest value was observed at the brackish water zone (Table 4-IV; Figure 4.4). On average, potential activity of esterase in the SML was similar (ANOVA $p>0.05$) to UW in both studied sites (marine zone average EF=1.1; brackish water zone average EF=1.5).

4.3.5.2.5.LIPASE

Lipase was the enzyme that showed lowest Hm values, varying between 0.5 and 9.7 nmol L^{-1} h^{-1} (Table 4-IV; Figure 4.4). The highest values were observed at the marine zone, where Hm of lipase was twice as high in the SML than in UW (average EF=2; ANOVA $p<0.05$). At the brackish water zone, the EF of lipase ranged from 0.9 to 3.6 (average 1.5), showing a modest enrichment.

4.3.6.CORRELATION ANALYSIS

The results of Spearman correlation between monomer incorporation (Vm) in the SML and UW, and between them and the other biotic and abiotic parameters are shown in the Table 4-V. The Vm incorporation of the different monomers in the SML and UW did not correlate significantly with each other, at both marine and brackish water zones. Significant correlations were between leucine incorporation in the SML and UW at both marine and brackish water zones were not observed. At the marine zone, Vm of glucose incorporation in the UW correlated significantly with the abundance of bacteria (TBN) and Chlorophyll a concentration. At the brackish water zone, bacterioplankton Vm incorporation of glucose correlated significantly with temperature and concentration of chlorophyll a. Vm incorporation of acetate enrichment factor at the marine zone correlated negatively with temperature.

Table 4-V. Spearman correlation between monomer incorporation (Vm), and between them and the other abiotic and biotic parameters in the SML and UW (SML/UW) at the marine (N1) and brackish water (I6) zones of the estuary Ria de Aveiro.

Spearman Correlation	Incorporation (Vm)		
	Leucine	Glucose	Acetate
	Marine zone [N1]		
SML/UW	*P*=0.027 (N.S) (n=11)	*P*=0.419 (N.S) (n=8)	*P*=0.286 (N.S) (n=10)
SML	N.S	N.S	N.S
UW	N.S	TBN- *P*=0.905 (n=8) (*) Chl a- *P*=0.857 (n=8) (**)	N.S
EF	N.S	N.S	Temp - *P*=-0.851 (n=10) (**)
	Brackish water zone [I6]		
SML/UW	*P*=0.388 (N.S) (n=11)	*P*=0.060 (N.S) (n=8)	*P*=0.438 (N.S) (n=10)
SML	N.S	N.S	N.S
UW	N.S	Temp- *P*=0.929 (n=8) (**) Chl a- *P*=0.786 (n=8) (*)	N.S
EF	EF TBN - *P*=0.664 (n=11) (*)	N.S	N.S

(**) Correlation is significant at the 0.01 level (two-tailed);
(*) Correlation is significant at the 0.05 level (two-tailed);
N.S. –Not significant;
Temp – Temperature; Chl a – Chlorophyll a; TBN – Total bacterial number;

The results of Spearman correlation between the different enzymatic activities in the SML and UW, and between them and the other biotic and abiotic parameters are shown in the Table 4-VI. At the marine zone, the activity of Leu-AMPase correlated with wind intensity at both compartments. In the UW of the brackish water zone, Hm of Leu-AMPase correlated positively with temperature. Hm of β-GlCase in the SML and UW correlated at the marine zone, and Hm of ß-GalCase correlated at the brackish water zone. In the UW of the marine zone, the activities of two enzymes were positively correlated. At the marine zone, Hm of β-GlCase in the SML and respective enrichment was correlated with wind intensity. In the UW of the brackish water zone, the Hm of ß-GalCase correlated with the fraction of particle-attached bacteria (% PAB). At the marine zone the enrichment of ß-GalCase Hm at the air-water interface correlated with the enrichment of % PAB and at the brackish water zone with particulate organic matter (POM). APase Hm in the SML and UW correlated at the marine zone but not at the brackish water zone. In the UW of both marine and brackish water zones, the activity of this enzyme correlated with the concentration of chlorophyll a and at the brackish water zone correlated also with PAB. In the SML of both estuarine zones, Hm of APase correlated with temperature and at the marine zone correlated also with wind intensity. The enrichment of APase activity at the air-water interface

correlated with temperature at the marine zone and with salinity at the brackish water zone. Esterase Hm in the SML and UW correlated at both estuarine zones. At the marine zone, the activity of this enzyme in the SML was positively correlated with the % PAB. In the UW of the brackish water zone, Hm of esterase correlated with POM. Hm of lipase correlated with total abundance of bacteria (TBN) in the UW of the brackish water zone.

Table 4-VI. Spearman correlation between the different enzymatic activities in the SML and UW (SML/UW) and between them and the others biotic and abiotic parameters at the marine [N1] and brackish water [I6] zones of the estuarine system Ria de Aveiro.

Spearman correlation	Enzymatic activities					
	Leu-AMPase	ß-GlCase	ß-GalCase	APase	Esterase	Lipase
			Marine zone [N1]			
SML/UW	P=0.983 (n=9) (**)	P=0.738 (n=8) (*)	P=0.552 (N.S) (n=10)	P=0.720 (n=10) (*)	P=0.714 (n=8) (*)	P=0.429 (n=8) NS
SML	Wind - P=0.717 (n=9) (*)	Wind - P=0.929 (n=8) (**)	NS	Temp- P=0.747 (n=10) (*) Wind - P=0.681 (n=10) (*)	% PAB - P=0.857 (n=7) (*)	NS
UW	Wind - P=0.636 (n=10) (*	ß-GalCase- P=0.738 (n=8) (*)	ß-GlCase- P=0.738 (n=8) (*)	Chl a- P=0.675 (n=10) (*)	NS	NS
EF	N.S	Wind - P=0.893 (n=7) (**)	EF % PAB - P=0.917(n=9) (**)	Temp - P=0.833 (n=9)(**)	NS	NS
			Brackish water zone [I6]			
SML/UW	P=0.882 (n=11) (**)	P=0.552 (n=10) NS	P=0.852 (n=10) (**)	P=0.500 (n=10) NS	P=0.750 (n=9) (*)	P=0.643 (n=8) NS
SML	NS	NS	NS	Temp- P=0.748 (n=10) (*)	NS	NS
UW	Temp- P=0.682 (n=11) (*)	N.S	% PAB - P=0.685 (n=10) (*)	PAB - P=0.833 (n=10) (**) Chl a- P=0.693 (n=10) (*)	POM - P=0.733 (n=9) (*)	TBN - P=0.762 (n=8) (*)
EF	NS	NS	POM - P=0.733 (n=10) (*)	Sal - P=0.767 (n=9) (*)	NS	NS

(**) Correlation is significant at the 0.01 level (two-tailed);
(*) Correlation is significant at the 0.05 level (two-tailed);
NS – Not significant;
Leu-AMPase – Leucine aminopeptidase; ß-GlCase – ß-glucosidase; GalCase – ß-galactosidase; APase – Alkaline Phosphatase; Sal - Salinity; Temp – Temperature; Chl a – Chlorophyll a; TBN – Total bacterial number; PAB – particle-attached bacteria (PAB); %PAB – fraction of PAB; POM – particulate organic matter;

4.4. DISCUSSION

Heterotrophic activities of neustonic and planktonic bacterial communities in the estuarine system Ria de Aveiro showed a well-defined pattern of variation. Generally, higher rates of hydrolysis and lower of monomer incorporation in the SML environment compared with UW were observed at the marine zone, whereas, at the brackish water zone, hydrolyze and incorporation rates were similar in the two compartments. This different pattern of variation of microbial activities at

the two contrasting estuarine sites might result from different organic and inorganic matter enrichments at the air-water interface, which reflect site-specific characteristics, such as hydrodynamics and organic matter composition.

4.4.1. HYDROLYTIC ACTIVITIES IN THE SML

Consistent and significantly higher hydrolysis rates of the enzymes ß-glucosidase, ß-galactosidase, alkaline phosphatase and lipase were observed in the SML at the marine zone of the estuary. Similar higher hydrolytic rates in the SML environment compared with UW were observed in other aquatic systems (Munster *et al.*, 1998; Kuznetsova & Lee, 2001; Mudryk & Skórczewski, 2004). Enhanced polymer hydrolysis in the SML is likely due to the accumulation of refractory DOM in the microlayer (Kuznetsova & Lee, 2001). In this work, the relation between hydrolysis rates observed in the SML and a potential DOM enrichment at the air-water interface of the marine zone cannot be confirmed because DOM was not analyzed. Nevertheless, a great variety of organic compounds of natural origin such as proteins, amino acids, carbohydrates, fatty acids, lipids, phenols, as well of anthropogenic origin concentrate at the air-water interface of diverse aquatic systems (Sieburth *et al.*, 1976; Hardy, 1982; Carlucci *et al.*, 1985; Carlucci *et al.*, 1986; Williams *et al.*, 1986; Carlucci *et al.*, 1991; Kuznetsova & Lee, 2002; Kuznetsova *et al.*, 2004). Therefore, the concentration of numerous organic and inorganic compounds in the SML can be assumed as a characteristic feature of this interface.

However, the degree of enrichment is dependent on the trophic status of the water column (Carlson, 1983; Wurl *et al.*, 2011). (Carlson, 1983) observed that DOM enrichments in the SML diminished with increasing concentrations in the UW. Additionally, (Wurl *et al.*, 2011) concluded that surfactant enrichment in the SML was higher in waters with lower than with higher primary production. The two studied sites have different concentrations of organic matter. The average concentration of chlorophyll a in the UW of the marine zone was 1.7 times significantly lower (ANOVA, $p<0.05$) than in the brackish water. Furthermore, in the UW of the brackish water zone, dissolved organic carbon (DOC) is on average, 3 times significantly (ANOVA, $p<0.05$) more concentrated than in the marine zone (data not shown). These different primary productivity and DOC concentration might result in higher enrichments of organic compounds in the SML of the marine than the brackish water zone. Therefore, microbial enzymatic activities in the SML of the estuarine system Ria de Aveiro may essentially reproduce the degree of organic matter enrichment and composition at the air-water interface.

Marine and brackish water zones of the estuary Ria de Aveiro have different amounts and prevalent sources of organic matter. At the marine zone, organic matter is predominantly of

autochthonous origin whereas at the brackish water zone it is a mix of allochthonous and autochthonous sources (Almeida *et al.*, 2005). Reflecting these different sources, amounts and composition of organic matter of the studied estuarine sites, the overall enzymatic activity increased at the brackish water zone of the estuary. However, the differences between the SML and UW were higher in the marine zone, particularly the activities of enzymes involved in the hydrolysis of carbohydrates and lipids, and phosphorus acquisition.

At the marine zone, the activity rates of the enzymes involved in the hydrolysis of carbohydrates (ß-glucosidase and ß-galactosidase) were, on average 7.3 and 3.6 times significantly higher in the SML compared with UW. This enhanced hydrolytic activity might be stimulated by a greater concentration of polysaccharides in the SML environment. The enrichment of polysaccharides in the SML appeared to be a common feature, with EFs ranging from 1.7 to 7.0 for particulate polysaccharides and 3.5 to 12.1 for polysaccharides in the HMW DOM fraction (Gao *et al.*, 2012). At the productive and hydrodynamic stable brackish water, the hydrolytic activity of ß-GlCase was only slight and statistically not significant enriched (EF=1.3), suggesting low substrate accumulation at the air-water interface of this estuarine zone.

Lipase activity was the lowest enzymatic activity detected in both marine and brackish water zones, reflecting the universally low concentration of lipids in aquatic systems (Harvey & Mannino, 2001). Despite the low concentration in aquatic systems, fatty acid lipids, *n*-alkanes, and total hydrocarbons are enriched in the SML by factors of 2-5 (Marty *et al.*, 1979). In the present study we observed a higher lipolytic activity in the SML, particularly at the marine zone of the estuary, suggesting a higher concentration of lipids at the air-water interface, perhaps due to their surface activity and/or high insolubility in water (Hunter, 1997). An enhanced activity of lipase was previously observed in this estuary and associated with anthropogenic sources of pollution, mainly due to fishery-related activities (Santos *et al.*, 2009). Due to its proximity to the harbor area, the marine zone is probably exposed to higher levels of pollution, namely hydrocarbons, which exhibit high accumulation at the air-water interface (Marty & Saliot, 1976; Hardy *et al.*, 1990; Garabetian *et al.*, 1993). A greater concentration of pollutants in the SML of the marine compared with brackish water zone could explain the different enrichments of lipolytic activity observed in the two zones of the estuary.

APase potential activity was always higher in the SML than in UW at both marine and brackish water zones of the estuary. The differences between the two compartments were more pronounced at the marine (6.6 times) than at the brackish water zone (2.1 times), suggesting a higher demand for phosphorus at marine zone of the estuary, particularly in the SML compartment. The marine zone presented always the lowest phosphate concentration (mean value <0.40 μmol L^{-1}) in the estuary (Lopes *et al.*, 2007a) and phytoplankton production increases 112 times at the

warm season compared with cold season (Almeida *et al.*, 2002a), which might result in an intense seasonal phosphorus demand and consequent increase of APase. At the brackish water zone, seasonal phytoplankton production variations are weaker (maximum 20.7 times) (Almeida *et al.*, 2002a) and consequently the variations of APase activity.

4.4.2. MONOMER UTILISATION IN THE ESTUARINE SML

An enhanced hydrolytic activity at the air-water interface might result in a higher concentration of easy assimilable monomeric compounds. However, at the marine zone of estuary, bacterioneuston communities showed unexpected lower rates of monomer incorporation compared with bacterioplankton. The potential of monomer incorporation by bacterioneuston was only 20 (leucine) and 30 (glucose and acetate) % of that of bacterioplankton at this particular zone of the estuary. This bacterioneuston inability for monomer utilization may be consequence of exposure to higher stress levels in the SML environment or/and unavailability of monomer to uptake.

Reduced potential heterotrophic activities of bacterioneuston compared with bacterioplankton were also reported by (Dietz *et al.*, 1976) and attributed to a greater stress that bacterial communities are exposed in the SML than in the water column. We recently (Santos *et al.*, 2011) showed that bacterioneuston biomass production (BBP) at marine zone was on average 10 times lower than bacterioplankton. The low rates of BBP in the SML were associated with the short residence time of bacterial communities as a consequence of the constant mix between the SML and water column, forced by the hydrodynamic characteristics at this estuarine area. Short residence times prevent bacterial adaptation and metabolic response to the potential organic enrichment of the air-water interface. Contrastingly, in the brackish water zone water currents are weak and the residence time is longer (Dias *et al.*, 2001). This hydrodynamic stability reduces vertical mixing, promoting stratification and increasing the residence time of the bacterial community at the surface and allowing the exploration of nutrients in the SML (Santos *et al.*, 2011), which might contributed to the similar neutonic and planktonic monomer incorporation rates observed at this specific estuarine zone.

Another possibility is a monomer-reduced availability to bacterioneuston due abiotic adsorption to colloidal DOM. (Schuster *et al.*, 1998) discovered that dissolved free amino acids (DFAA) readily adsorb to colloidal DOM and particularly to polysaccharides, reducing their availability by 2 to 3 orders of magnitude. Considering the gelatinous nature of the SML film, mainly constitute by carbohydrates (Sieburth, 1983; Wurl & Holmes, 2008), the hydrolyze products might adsorb to the organic matrix reducing their availability to bacteria, which could explain the low rates of monomer incorporation observed in this particular environment. On the other hand,

adsorption of DFAA to colloidal matter increases the accessibility of colloidal matter to bacterial degradation (Schuster *et al.*, 1998). The impact of monomer adsorption to colloidal matter and consequent increases of accessibility to microbial enzymatic degradation might be higher in the marine zone, where potentially occur a higher accumulation of organic matter at the air-water interface as a consequence of the low productivity of the water column.

4.5. CONCLUSION

In Ria de Aveiro, the differences between hydrolysis and monomer incorporation rates by bacterioneuston and bacterioplankton communities might result from the different amounts and composition of organic matter and the hydrological characteristics of the estuary. At the marine zone, the less productive water column and the strong hydrological forced mix promote intense hydrolytic activities and low monomer utilization. At the brackish water zone, a more productive water column and a higher hydrodynamic stability, lead to similar bacterioneuston and bacterioplankton heterotrophic activities. The degree of differentiation between heterotrophic activities of bacterial communities in the SML and UW in estuarine systems is, therefore, a result of the different organic enrichments at the air-water interface and the intensity of exposure to physical processes

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CHAPTER 5

5. PHOTOCHEMICAL AND MICROBIAL ALTERATIONS OF DOM SPECTROSCOPIC PROPERTIES IN THE ESTUARINE SYSTEM RIA DE AVEIRO

Abstract

The influence of photochemical transformations of colored dissolved organic matter (CDOM) on microbial communities was evaluated in the estuarine system Ria de Aveiro. Two sites, representative of the marine and brackish water zones of the estuary were surveyed regularly in order to determine seasonal and vertical profiles of variation of CDOM properties. Optical parameters of CDOM indicative of aromaticity and molecular weight were used to establish CDOM sources and, microbial abundance and activity was characterized. Additionally, microcosm experiments were performed in order to simulate photochemical reaction of CDOM and to evaluate microbial responses to light-induced changes in CDOM composition. The CDOM of estuarine zones showed different spectral characteristics, with significant higher values of the specific ultra-violet absorbance at 254 nm ($SUVA_{254}$) (5.5 times) and of the absorption coefficient at 350 nm (a_{350}) (12 times) and lower ratio S_R ($S_{275-295}/S_{350-400}$) at brackish water compared with the marine zone, reflecting the different amounts and prevailing sources of organic matter, as well as distinct riverine and oceanic influences. At the marine zone, the abundance of bacteria and the activity of Leu-AMPase correlated with a_{350} and a_{254}, suggesting a microbial contribution to HMW CDOM pool. The irradiation of DOM resulted in a decrease of the values of a_{254} and a_{350} and in an increase of the slope $S_{275-295}$, and of the ratios E_2:E_3 (a_{250}/a_{365}) and S_R, which in turn increase its bioavailability. However, the extent of photoinduced transformations and microbial responses was dependent on the initial optical characteristics of CDOM. In Ria de Aveiro both photochemical and microbial processes yielded optical changes in CDOM and overall result of these combined processes determine the fate of CDOM in the estuarine system and have influence on local productivity and in adjacent coastal areas.

Keywords: CDOM; photochemical; microbial activity; estuary; Ria de Aveiro;

5.1. INTRODUCTION

The concentration, quality/composition of dissolved organic matter (DOM) and dissolved inorganic nutrients are key variables that influence the size, distribution and metabolism of bacterial communities in aquatic ecosystems (Eiler *et al.*, 2003; Kirchman *et al.*, 2004; Apple & del Giorgio, 2007), namely in estuaries (Cunha *et al.*, 2000; Almeida *et al.*, 2005; Barrera-Alba *et al.*, 2009; Cunha & Almeida, 2009). The light-absorbing fraction of DOM, chromophoric dissolved organic matter (CDOM), from terrestrial and autochthonous origins, is the primary absorber of sunlight in aquatic ecosystems and plays an important role for most photochemically mediated processes in surface waters (Mopper & Kieber, 2002).

Natural solar radiation, especially ultraviolet radiation (UV-B [280–315 nm], UV-A [315–400 nm]), has been found to induce chemical transformations of CDOM with the production of a variety of photoproducts, including inorganic carbon (Johannessen & Miller, 2001; Zhang *et al.*, 2006; White *et al.*, 2010), nitrogen (Bushaw *et al.*, 1996; Smith & Benner, 2005; Stedmon *et al.*, 2007) and phosphorus (Vähätalo *et al.*, 2003) compounds, and numerous low molecular weight (LMW) organic compounds (Wetzel *et al.*, 1995; Bertilsson & Tranvik, 1998, 2000), which in turn could stimulate bacterial metabolism (Bushaw *et al.*, 1996; Bano *et al.*, 1998; McCallister *et al.*, 2005). The origin and chemical composition of DOM strongly influences its photoreactivity (Obernosterer *et al.*, 1999; Tedetti *et al.*, 2009) and photoproduction of dissolved inorganic carbon (DIC) (Minor *et al.*, 2007) and LMW organic compounds (Kieber *et al.*, 1990; de Bruyn *et al.*, 2011) has been shown to correlate with the concentration of UV-absorbing CDOM, measured by the absorbance.

Optical properties of CDOM have been used to characterise sources, composition and diagenic stage of DOM in a wide range of aquatic ecosystems (Stedmon & Markager, 2001; Spencer *et al.*, 2007b; Spencer *et al.*, 2007a; Helms *et al.*, 2008; Spencer *et al.*, 2009; Stephens & Minor, 2010), as well as induced photochemical transformations (Moran *et al.*, 2000; Helms *et al.*, 2008; Zhang *et al.*, 2009). Based on the finding of Helms et al (2008), and with a recent increasing application, the spectral slope of a narrow wavelength interval between 275 and 295 nm region ($S_{275\text{-}295}$), has been use to trace terrestrial DOM (Fichot & Benner, 2012; Lin *et al.*, 2012; Fichot *et al.*, 2013). Additionally, the ratio between this and the slope between 350 and 400 nm region ($S_{350\text{-}400}$), S_R, provide information about DOM MW and photochemically induced alterations (Helms *et al.*, 2008; Zhang *et al.*, 2009).

This study aimed to characterise seasonal profiles of variation of CDOM in Ria de Aveiro, as well as to evaluate the influence of photochemical and microbial processes in the dynamics of this light absorbing component of DOM in the estuarine system. In order to achieved that goal, selected CDOM optical properties, related with aromaticity and MW, were correlated with

microbial parameters and photochemical induced transformations were experimentally simulated.

5.2. METHODS

5.2.1. SAMPLING STRATEGY AND LABORATORY SIMULATIONS

In a first stage, two estuarine sites (stations N1 and I6; Figure 5.1) were sampled regularly, in order to identify the main sources and characterise DOM in the Ria de Aveiro, as well as the temporal and spatial profiles of variation. The two sites display distinct levels of microbial activity (Cunha *et al.*, 2000; Almeida *et al.*, 2001c; Almeida *et al.*, 2002b), amounts of organic matter and are differently impacted by river discharges and oceanic influence. Station N1, located near the mouth of estuary, is highly exposed to oceanic influence, whereas, station I6, located at inner section of the Ílhavo channel, the narrower and shorter of the main channels (Dias *et al.*, 2001), is directly influenced by river Boco discharge. The two sites have also different water columns dimensions and transparency and, are currently referred as the marine zone (MZ) and brackish water (BZ) zones, respectively (Almeida *et al.*, 2001a; Almeida *et al.*, 2002b). In a second stage, in order to understand the influence of photochemical processes in the estuarine system, particularly in the deep and transparent areas, which are highly impacted by solar radiation, water samples from the station N1 were irradiated to sunlight irradiation during long-term (168 h). The response of estuarine bacteria to irradiated DOM was evaluated as well by the determination of bacterial biomass production. Additionally, these experiments allowed the determination of the most convenient timeframe to assess measurable photochemical alterations of DOM induced by sunlight irradiation and bacterial growth. Therefore, in a third stage, short-term experiments (12h) were performed and the response of bacteria to irradiated DOM was characterized in detail.

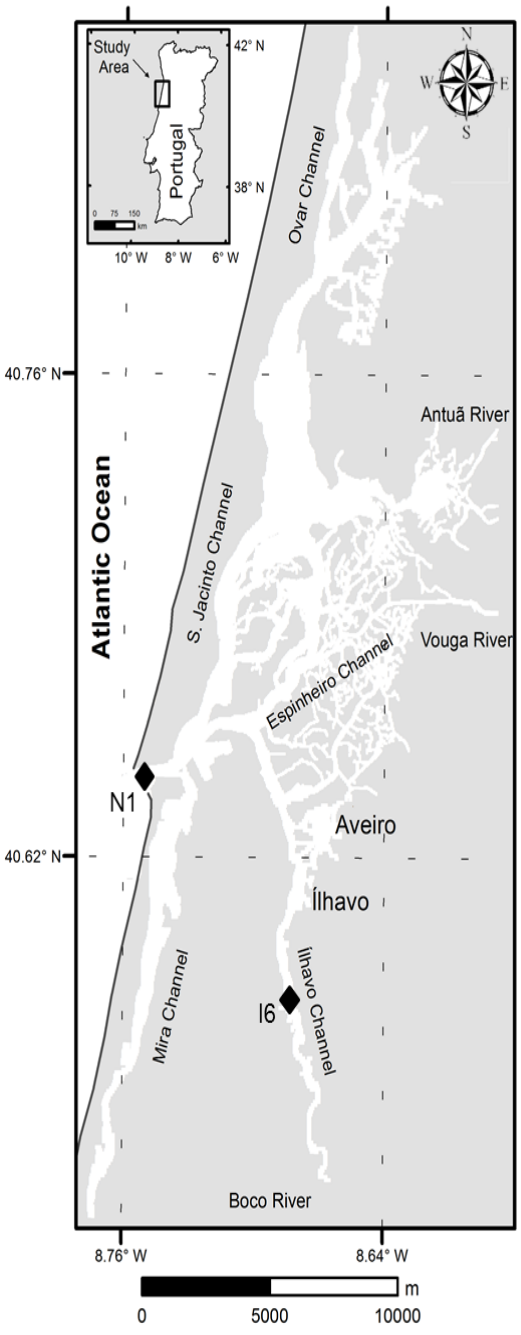

Figure 5.1. The estuarine system Ria de Aveiro with indication of sampling stations. Station N1 in Canal de Navegação represents the marine zone, and station I6, in Canal de Ílhavo, represents the brackish water zone.

5.2.2. STUDY SITE AND SAMPLING

Ria de Aveiro (40º 38'N, 8º 45'W; Figure 5.1) is a shallow tidal lagoon (Dias *et al.*, 2000) situated on the Northwest Atlantic coast of Portugal, separated from the sea by a sand bar. The lagoon covers an area ranging from 66 at low tide to 83 km^2 at high tide. It exchanges with the sea a volume of water of 137 Mm^3 for maximum spring tide and 35 Mm^3 for minimum neap tide (Dias *et al.*, 2000). The lagoon has a complex topography, with four main channels spreading from the mouth: S. Jacinto, Espinheiro, Mira and Ílhavo. Due to their unique characteristics, each channel could be considered as an independent estuary connected to a common inlet (Dias *et al.*, 2001). Freshwater is supplied to lagoon mainly by rivers Vouga, Antuã, Caster, Gonde and Boco, which discharge an average water input of 1.8 Mm^3 during a tidal cycle (Dias *et al.*, 2003). Of these rivers, the major contributor is Vouga River which discharges more than 66% of the incoming freshwater (Dias *et al.*, 1999) and is connected to the Atlantic Ocean by the Espinheiro Channel. Sampling was conducted at low tide, approximately every two months, between January 2006 and April 2007, at station N1 and I6. Water samples were collected with a horizontal Van Dorn bottle at the fixed depths of 20 cm, 50 cm, mid-depth and 50 cm above the sediment surface. Samples were kept at 4 ºC during the transport to the laboratory and processed within 2-3 hours after collection.

5.2.3. WATER COLUMN PROPERTIES

Water temperature and salinity were measured in the field using a WTW LF 196 Conductivity Meter (Wissenschaftlich Technische Werkstätten). The total depth of the water column was estimated with a Sonar probe (Hondex PS-7 LCD Digital Sounder). Chlorophyll a (Chl a) was estimated fluorimetrically (Yentsch & Menzel, 1963) after filtration of 0.5 L triplicate subsamples through Whatman GF/F filters and overnight cold extraction in 90% (v/v) acetone. Suspended particulate matter (SPM) concentration was determined after filtration of triplicate 0.5 L water aliquots through pre-weighted and pre-combusted Whatman GF/F filters. The filters were dried at 60 ºC for 24 h, and SPM was calculated as the increase in dry weight. Particulate organic matter (POM) was determined from loss of weight after 4 h incineration at 550°C (Parsons *et al.*, 1989). For nutrient analysis, water subsamples were filtered through MSI acetate membranes (GE Osmonics) with 0.45 µm pore size and stored at -20 °C in acid-cleaned polyethylene flasks until determination. Orthophosphate and nitrite were quantified using methods described by Hansen and Koraleff (2007). Nitrate was assayed using an adaptation of the spongy cadmium reduction technique (Jones, 1984), with the nitrite value subtracted from the total. The concentrations of total carbon (TC) and inorganic carbon (IC) were determined with a Shimadzu TOC-5000A analyzer.

The dissolved organic carbon (DOC) content of the samples was calculated as the difference TC − IC. For TC quantification, standards were prepared from reagent grade potassium hydrogen phthalate (Fluka) in ultrapure water in the range of 0.5 to 2 mg C L^{-1}. For IC measurements, standards were made from reagents grade sodium hydrogenocarbonate (Fluka) plus sodium carbonate (Fluka) in ultrapure water, also in the range of 0.5 to 2 mg C L^{-1}. Control standards were generally within 5% agreement in terms of TC and IC content. For each sample, three replicates were analyzed for determining the DOC content.

5.2.4. MICROCOSM EXPERIMENTS

5.2.4.1. EXPERIMENTAL SET-UP AND TREATMENTS

Water samples for the short (12 h irradiation) and long-term (168 h irradiation) assays experimental set-up treatments were collected at the N1 station, at low tide, with a horizontal Van Dorn bottle at 20 cm depth, in May and June 2006, for long-term assays, and in May 2009, for the short-term assay. Water samples were filtered sequentially through pre-combusted (550°C, 4 h) Whatman GF/F filters (0.7 μm) and then through 0.2 μm PVDF filters (Pall Corporation) in order to remove POM and bacteria. Filtered water was stored at 4°C in the dark for 12 h prior to the incubation.

Long-term experiments: For long-term experiments, 3 L of bacteria-free filtrate (0.2 μm filtrate) was distributed by 150 mL quartz tubes (natural sunlight-irradiated sample) and 1L aluminum foil-wrapped borosilicate bottles (dark control). Both quartz tubes and dark controls were irradiated with natural sunlight at environment temperature conditions during 168 h. Replicates for DOM spectral characterization (UV/Vis spectroscopy) and bioassays were collected at 0, 12 and 168 h. Bioassays were initiated by addition of a natural bacterial inoculum that was obtained from the same study site (0.7 μm filtrate) diluted 10-fold. Incubations were conducted in the dark at room temperature (22°C) during 168 h, with gentle agitation, and subsamples were collected at 0, 72 and 168 h in order to assess the response of bacterial biomass production to the different periods of DOM irradiation with sunlight. DOM characterization and determinations of bacterial production were performed immediately.

Short-term experiments: For the short-term experiments, 3 sub-samples with 2.5 L of bacterial free filtrate (0.2 μm filtrate) were placed in 3L Pyrex trays (natural sunlight treatment). The trays were wrapped in polypropylene foil, which screens out approximately 20% of PAR, 25 % of UVR-A and 30 % of UVR-B, and 3L borosilicate bottles were wrapped in aluminum foil representing dark controls. Pyrex trays and dark controls were irradiated with natural sunlight and environment temperature conditions during 12 h, in a cloudless day. Optical characteristics of

DOM (UV/Vis spectroscopy) were determined before and after exposition to natural sunlight. Bioassays were initiated by adding a natural bacterial inoculum (0.7 μm filtrate) obtained from the same study site and diluted 10-fold. Incubations were conducted in the dark at room temperature (22°C) during 108 h, with gentle agitation. Subsamples were collected at 0, 72 and 108 h to assess the response of bacteria to irradiated DOM.

5.2.5. CDOM SPECTROSCOPIC CHARACTERISTICS

UV–Vis spectroscopy was performed on a Shimadzu Model UV 210PC spectrophotometer using 1 and 10 cm quartz cuvettes (as required depending on sample absorptivity) for the range 200–700 nm. The absorption coefficients (a_λ, m^{-1}) at each wavelength (λ) were calculated as $a_\lambda = 2.303\ A_\lambda/l$, where A_λ is the absorbance reading at wavelength λ and l (m) is the optical path length (Green & Blough, 1994). Ultrapure water was used as standard and to obtain the baseline. Following blank correction, the absorbance of a given sample at the wavelength 700 nm was subtracted from each absorbance value in the 200–700 nm range.

The absorption coefficients (a) were used in the determination of the spectral characteristics listed below. The ratio of spectral slopes, S_R, is the ratio of S values for wavelengths 275–295 nm ($S_{275–295}$) and 350–400 nm ($S_{350–400}$) where the values for these S sections were determined by plotting ln(a) versus wavelength (Helms *et al.*, 2008). The E_2:E_3 ratio is calculated as the ratio of a_{250} to a_{365} and is inversely correlated with molecular size (Peuravuori & Pihlaja, 1997). Specific ultra-violet absorbance at 254 nm ($SUVA_{254}$) was calculated by dividing a_{254} by the DOC concentration in mg L^{-1}. $SUVA_{254}$ is indicative of the amount of humification or aromaticity within the sample (Weishaar *et al.*, 2003).

5.2.6. TOTAL BACTERIAL NUMBER

Total bacterial number (TBN) was determined by epifluorescence microscopy using a Leica DMLS microscope equipped with a I 2/3 filter for blue light. Three replicates for each sample were filtered through 0.2 μm black polycarbonate membranes (GE Osmonics) and stained with 0.03 % acridine orange (Hobbie *et al.*, 1977). At least 200 cells or 20 microscope fields were counted for each replicate measurement.

5.2.7. BACTERIAL BIOMASS PRODUCTION

Bacterial biomass production (BBP) was determined in 10-ml triplicate plus a control that was fixed by addition of formaldehyde (2% final concentration). The samples were incubated at a saturating concentration (121.6 nM) of ^{3}H-leucine (Amersham, specific activity - 2.55 TBq mmol $^{-1}$) for 1 h, at *in situ* temperature, in the dark. After incubation, replicates were fixed with 2% (v/v) formaldehyde. Protein was precipitated by the addition of 1 ml of 20% (w/v) ice-cold TCA followed by incubation for 15 min on ice. The 10-ml triplicate and the control were then filtered through 0.2 μm polycarbonate membranes (GE Osmonics) and rinsed with 2 ml of 5% (w/v) ice-cold TCA and 5 ml of 90% (v/v) ice-cold ethanol. Membranes were then placed into 5 mL scintillation vials and 4.5 mL of scintillation cocktail UniverSol (ICN Biomedicals, USA) was added. Radioactivity was measured after a period of 3 days in a Beckman LS 6000 IC liquid scintillation counter. BBP was calculated from leucine incorporation rates using a ratio of cellular carbon to protein of 0.86 and a fraction of leucine in protein of 0.073 (Simon & Azam, 1989).

5.2.8. EXTRACELLULAR ENZYMATIC ACTIVITY

Extracellular enzymatic activity **(**EEA) was determined fluorimetrically (Jasco FP-777 fluorometer) as the maximum hydrolysis rate (Hm) of model substrates (Hoppe, 1991). The substrate L-leucine-7-amido-4-methyl-coumarin hydrochloride (Fluka) was used for leucine aminopetidase (E.C. 3.4.11.1) (Kanaoka *et al.*, 1977) and the 4-methylumbelliferyl-β-glucopyranoside (Fluka) for β-glucosidase (E.C. 3.2.1.21) (Daniels *et al.*, 1981). Both substrates were added at saturating concentrations (10 mM). Wavelengths for excitation and emission were 380 to 440 nm for MCA (7-amino-4-methylcoumarine) and 360 to 450 nm for MUF (4-methylumbelliferone). Measurements were made in 3 replicates for each sample after 2 h, for MCA, and 18 h for MUF. Incubations were made at *in situ* temperature. Calibration was performed by adding a series of 6 to 8 concentrations of the fluorescent products (0 to 500 nM for MUF and 0 to 6 μM for MCA) to a pool of water from the 2 sampling stations.

5.2.9. DATA ANALYSIS

The statistical analysis of data was performed with the SPSS 15.0 (SPSS Statistics) software. The relations between the different parameters were examined using a Spearman correlation. One-Way ANOVA was used to determine the significance of the differences observed in microbial and spectral parameters at the two estuarine zones and at the different depths. Normal distribution was assessed by the Kolmogorov-Smirnov test and the homogeneity of variances by the Levene test. A value of $p<0.05$ was considered significant.

5.3.RESULTS

5.3.1.WATER PROPERTIES

5.3.1.1.PHYSICOCHEMICAL PARAMETERS

The values of the physicochemical parameters determined in the water column at the marine (station N1) and brackish (station I6) water zones of Ria de Aveiro are summarized in Table 5 - I. Salinity ranged from 10.1 to 36.5 psu (average 31.6 ± 6.68 psu) at station N1 and from 0.2 to 36.5 psu (average 18.9 ± 12.73 psu) at station I6. Water temperature varied between 12.5 and 19.6 ºC (average 16.0 ± 2.31 ºC) at station N1, and between 10.2 and 24.5 ºC (average 17.6 ± 5.38 ºC) at station I6. The average concentration of nitrate plus nitrite (NO_3^- + NO_2^-) was 7.2 ± 3.70 µM (2.2 - 19.4 µM) at station N1 and 34.9 ± 24.54 µM (4.2 – 92.3 µM) at station I6. Phosphate (PO_4^{3-}) concentration varied between 0.1 and 38.6 µM (average 4.5 ± 9.14 µM) at station N1 and, between 0.4 and 50.4 µM (average 6.5 ± 13.33 µM) at station I6. Chlorophyll a concentration ranged from 1.7 to 6.0 µg L^{-1} (average 3.4 ± 1.41 µg L^{-1}) at station N1and from 2.5 to 10.1 µg L^{-1} (average 5.4 ± 2.39 µg L^{-1}) at station I6. Suspended particulate matter (SPM) concentration varied between 26.1 and 178.1 mg L^{-1} (average 61.0 ± 27.00 mg L^{-1}), and between 28.5 and 83.1 mg L^{-1} (average 52.8 ± 18.58 mg L^{-1}) at stations N1 and I6, respectively. The concentration of particulate organic matter (POM) was, on average, 15.1±5.36 mg L^{-1} (6.9 - 33.7 mg L^{-1}) at station N1 and 12.1±4.90 mg L^{-1} (5.3 – 20.0 mg L^{-1}) at the brackish water zone. The average concentration of dissolved organic carbon (DOC) was 4.3±9.14 mg L^{-1} (0.5 – 13.9 mg L^{-1}) at station N1 and 10.9±6.40 mg L^{-1} (2.1 – 26.0 mg L^{-1}) at station I6.

5.3.1.2.SPECTRAL CHARACTERISTICS OF CDOM

The values of the different spectral characteristics of CDOM determined in the water column at the station N1 and station I6 are showed in Table 5-II. In Ria de Aveiro, the average value of $SUVA_{254}$ was 5.5 times significantly higher (ANOVA, $p<0.05$) at the brackish water zone compared with marine zone, ranging from 0.31 to 62.4 L mg^{-1} C m^{-1} (average 6.7±12.11 L mg^{-1} C m^{-1}). The absorption coefficient at 350 nm (a_{350}) varied between 0.16 and 41.30 m^{-1} and was 12 times significantly higher (ANOVA, $p<0.05$) at the brackish water zone compared with the marine zone. At station N1, the average value of $S_{275-295}$ was 0.0073±0.00142 nm^{-1} and, at station I6 was 0.0065±0.00068 nm^{-1}. The differences between the two estuarine zones were statistically significant (ANOVA, $p<0.05$). Ranging from 0.0019 and 0.0102 nm^{-1}, the average values of $S_{350-400}$ at the marine (0.0064±0.00173 nm^{-1}) and brackish water (0.0075±0.00082 nm^{-1}) zones were statistically different (ANOVA, $p<0.005$). The ratio E_2:E_3 was similar at both estuarine zones (ANOVA, $p>0.05$), ranging from 1.9 to 8.9 (average 5.9±1.05). The ratio SR ranged from 0.77 to 4.19 (average 1.25±0.627) at station N1 and, from 0.71 to 1.28 (average 0.89±0.119) at the station

I6. The differences between the two stations were statistically significant (ANOVA, $p<0.05$). All DOM spectral characteristics were similar (ANOVA, $p>0.05$) at the different sampling depth in the water column of both estuarine zones.

Table 5-I. Water column physical and chemical characteristics in the different sampling events at the marine (station N1) and brackish water (station I6) zones of the estuarine system Ria de Aveiro.

Year	2006						2007	
Month	Jan	Mar	May	Jul	Sep	Dec	Feb	Apr
Marine zone [N1]								
Sal [psu]	33.4±0.36	28.1±5.72	34.6±0.53	36.4±0.10	35.5±0.13	22.2±13.03	28.6±5.98	34.1±0.29
Temp [ºC]	12.6±0.10	15.1±0.25	18.0±0.23	17.7±0.36	19.4±0.17	14.4±1.62	14.1±0.13	16.8±1.45
$NO_3^- + NO_2^-$ [µM]	10.4±1.68	7.5±2.10	4.9±0.93	7.4±2.17	6.6±1.79	12.4±6.44	3.4±0.82	5.2±0.83
PO_4^{3-} [µM]	1.3±0.21	1.0±0.14	1.0±0.09	1.6±0.64	2.2±0.54	26.9±9.59	1.7±1.11	0.2±0.09
Chl a [µg L^{-1}]	2.1±0.24	2.6±0.72	3.5±0.53	5.7±0.27	2.0±0.16	2.6±1.34	4.0±0.52	4.9±0.56
SPM [mg L^{-1}]	46.5±1.73	44.5±8.20	61.1±4.20	72.3±3.79	59.2±4.52	83.8±71.66	56.6±19.88	64.4±17.87
POM [mg L^{-1}]	12.0±0.46	11.8±2.31	14.8±1.32	20.9±1.85	17.0±0.94	17.3±12.86	15.3±4.37	12.0±1.79
DOC [mg L^{-1}]	2.4±0.69	7.0±3.79	2.4±0.85	9.2±3.21	N.D	1.0±0.41	4.1±2.47	4.2±2.32
Brackish water zone [I6]								
Sal [psu]	20.7±0.21	7.8±0.26	27.1±0.15	36.5±0.06	31.6±0.85	0.3±0.06	3.9±0.13	23.2±0.10
Temp [ºC]	10.9±0.18	16.6±0.10	23.5±0.45	24.3±0.18	21.9±0.21	10.2±0.13	13.5±0.05	20.3±0.25
$NO_3^- + NO_2^-$ [µM]	47.7±5.54	75.6±17.75	25.7±5.26	12.8±3.58	19.5±6.95	13.7±7.02	58.9±27.22	25.3±2.11
PO_4^{3-} [µM]	0.9±0.28	1.7±0.27	1.6±0.48	2.9±0.32	4.2±0.88	40.4±12.22	3.6±0.47	0.6±0.17
Chl a [µg L^{-1}]	2.8±0.33	4.3±0.37	9.7±0.32	6.3±1.14	3.5±0.24	3.2±0.19	5.7±0.65	7.9±0.26
SPM [mg L^{-1}]	30.7±1.30	37.5±2.34	71.1±2.13	74.0±4.30	59.9±4.30	29.7±0.81	49.2±1.20	70.5±17.11
POM [mg L^{-1}]	8.0±0.61	7.5±0.32	14.2±1.10	19.1±1.21	18.0±1.60	5.4±0.16	10.8±0.64	13.6±2.78
DOC [mg L^{-1}]	11.6±2.86	14.7±3.04	8.5±1.92	22.7±3.41	N.D	4.3±2.35	4.8±2.20	10.1±2.18

Average of the water column ±standard deviation (N=4); Sal - salinity; Temp - Temperature; Chl a - Chlorophyll a; SPM – Suspended particulate matter; POM - Particulate organic matter; DOC - Dissolved organic carbon;

Table 5-II. Water column physical and chemical characteristics in the different sampling events at the marine (station N1) and brackish water (station I6) zones of the estuarine system Ria de Aveiro.

Year	2006						2007	
Month	Jan	Mar	May	Jul	Sep	Dec	Feb	Apr
				Marine zone [N1]				
$SUVA_{254}$ [L mg^{-1} C m^{-1}]	1.74±0.544	2.52±3.058	3.05±4.690	1.77±1.266	3.21±2.323	0.67±0.184	N.D	1.37±1.120
a_{350} [nm^{-1}]	0.753±0.039	1.213±0.723	1.995±1.379	0.931±0.257	1.447±0.701	1.243±0.201	0.966±0.108	0.372±0.142
$S_{275-295}$ [nm^{-1}]	0.009±0.0002	0.008±0.0010	0.008±0.0014	0.008±0.0002	0.006±0.0012	0.007±0.0004	0.008±0.0003	0.005±0.0016
$S_{350-400}$ [nm^{-1}]	0.007±0.0003	0.009±0.0009	0.007±0.0003	0.003±0.0011	0.006±0.0003	0.006±0.0007	0.007±0.0006	0.006±0.0025
E_2:E_3	6.60±0.328	7.43±1.344	5.92±1.061	5.85±0.841	6.11±0.397	6.35±0.465	6.76±0.584	3.74±1.432
S_R	1.31±0.045	0.84±0.059	1.11±0.194	2.52±1.126	0.96±0.178	1.08±0.080	1.25±0.073	0.96±0.199
				Brackish water zone [I6]				
$SUVA_{254}$ [L mg^{-1} C m^{-1}]	3.21±0.656	24.37±14.115	5.40±0.995	3.04±0.621	3.44±0.718	1.12±0.162	N.D	38.35±19.849
a_{350} [m^{-1}]	10.08±1.944	23.84±1.944	19.25±0.368	6.48±0.184	6.29±0.662	5.33±0.216	5.36±0.076	35.01±4.208
$S_{275-295}$ [nm^{-1}]	0.006±0.0005	0.006±0.0000	0.006±0.0001	0.007±0.0001	0.007±0.0002	0.007±0.0001	0.007±0.0000	0.006±0.0001
$S_{350-400}$ [nm^{-1}]	0.006±0.0010	0.008±0.0001	0.008±0.0001	0.008±0.0001	0.007±0.0004	0.007±0.0002	0.008±0.0001	0.008±0.0004
E_2:E_3	4.52±0.646	5.44±0.061	5.44±0.028	6.38±0.188	6.06±0.310	6.53±0.138	5.96±0.076	5.23±0.211
S_R	1.03±0.164	0.76±0.007	0.80±0.011	0.89±0.011	0.98±0.011	0.97±0.011	0.93±0.015	0.73±0.015

5.3.2. BACTERIOPLANKTON: ABUNDANCE, BIOMASS PRODUCTIVITY AND EXTRACELLULAR ENZYMATIC ACTIVITY

The values of total bacterial number (TBN), bacterial biomass production (BBP) and, the activity rates of the enzymes aminopepetidase (Leu-AMP) and ß-glucosidase (ß-GlCase) determined in the water column at station N1 and station I6 are presented in Table 5-III. TBN varied between 0.5 and 12.6 x 10^9 cells L^{-1} and, the average value was 1.7 times significantly higher (ANOVA, $p<0.05$) at the station I6 (average 4.6±2.44 10^9 cells L^{-1}) compared with station N1 (average 2.7±2.07 10^9 cells L^{-1}). BBP was similar at both estuarine zones (ANOVA, $p>0.05$), ranging from 0.6 to 32.1 µg C L^{-1} h^{-1} (average 7.2±7.52 µg C L^{-1} h^{-1}) at the station N1and from 2.6 to 26.8 µg C L^{-1} h^{-1} (average 8.4±5.07 µg C L^{-1} h^{-1}) at the station I6. The average Hm of Leu-AMPase value was similar (ANOVA, $p>0.05$) at the station N1 (2686±3750.3 nmol L^{-1} h^{-1}) and station I6 (3412±3153.6 nmol L^{-1} h^{-1}). Hm of ß-GlCase values ranged from 0.9 to 2373.3 nmol L^{-1} h^{-1}, and were, on average, 3.3 times lower at the station N1 (132.1±152.62 nmol L^{-1} h^{-1}) compared

with the station I6 (431.4±661.16 nmol L^{-1} h^{-1}). The values of different microbiological descriptors were similar (ANOVA $p>0.05$) at the different sampling depths in the water column at the two studied estuarine zones.

Table 5-III- Total bacterial number (TBN), bacterial biomass production (BBP) and, hydrolysis rates of the enzymes aminopeptidade (Leu-AMPase) and ß-glucosidase (ß-GlCase) in the water column at the marine and brackish water zones of the estuarine system Ria de Aveiro. Average of the water column ± standard deviation (N=4).

Year	2006						2007	
Month	Jan	Mar	May	Jul	Sep	Dec	Feb	Apr
Marine zone [N1]								
TBN [x10^9 cells L^{-1}]	2.1±0.25	3.5±1.37	4.4±1.43	6.7±0.66	2.4±0.41	1.4±0.35	0.5±0.04	0.8±0.11
BBP [µg C L-1 h-1]	0.7±0.07	3.2±1.33	3.7±0.54	6.3±2.03	8.3±0.53	6.1±2.51	6.3±1.36	23.3±11.21
Leu-AMPase [nmol L^{-1} h^{-1}]	607±27.1	1501±247.3	3214±324.4	12134±1127.8	792±84.7	997±131.3	330±127.0	1910±195.0
ß-GlCase [nmol L^{-1} h^{-1}]	N.D	70.9±44.71	74.7±15.19	309.7±53.79	26.4±26.79	359.6±208.02	10.2±4.19	73.3±27.93
Brackish water zone [I6]								
TBN [x10^9 cells L^{-1}]	4.6±1.15	4.4±0.57	3.8±0.61	10.3±1.56	4.3±0.43	4.5±0.31	2.5±0.60	2.3±0.20
BBP [µg C L-1 h-1]	2.8±0.22	4.9±1.39	7.5±1.15	8.5±0.86	8.7±0.56	10.0±2.05	13.1±11.83	11.7±3.25
Leu-AMPase [nmol L^{-1} h^{-1}]	2560±259.4	2363±25.4	4925±123.3	11001±338.6	1631±139.7	972±35.7	1020±73.5	2827±142.2
ß-GlCase [nmol L^{-1} h^{-1}]	11.8±2.64	72.2±15.03	278.2±193.42	555.2±396.73	245.3±146.54	2036.8±301.1	85.9±19.29	165.9±122.90

5.3.3. CORRELATIONS OF CDOM SPECTRAL PROPERTIES WITH ENVIRONMENTAL CHARACTERISTICS

The Spearman correlations between spectral parameters of CDOM and the different physicochemical parameters in the water column of the station N1 and station I6 are shown in Table 5-IV. All of the investigated spectral properties showed higher number of correlations with the environmental variables in the water column at the station I6 compared with station N1. The concentrations of chlorophyll a and POM were negatively correlated with the $SUVA_{254}$ at both estuarine zones. $SUVA_{254}$ also showed a negative correlation with salinity, particularly at the station I6. At this particular zone, $SUVA_{254}$ also correlated negatively with temperature. The absorption coefficient at 350 nm (a_{350}) at station N1 only showed a strong correlation with the absorption coefficient at 254. However, at the station I6, this specific coefficient correlated negatively with POM and DOC concentrations, and with salinity and temperature. The concentration of nitrate plus nitrite showed a positive correlation with this property of CDOM. At

station N1, the ratios E_2:E_3 and S_R and, the slope $S_{275\text{-}295}$ did not correlated with any of the physicochemical parameters. However, these spectral characteristics at the station I6 were correlated positively with the concentrations of POM and chlorophyll a and, with temperature and salinity. The concentration of nitrate plus nitrite correlated negatively with the ratio E_2:E_3 and the slope $S_{275\text{-}295}$. The slope $S_{350\text{-}400}$ correlated negatively with SPM and chlorophyll a concentrations at the station N1and, DOC concentration and salinity at the station I6.

Table 5-IV. Spearman correlations between dissolved organic matter (DOM) spectroscopic parameters and the different chemical and physical parameters at the marine and brackish water zones of the estuary Ria de Aveiro.

	Marine zone [N1]	*Brackish water zone [I6]*
$SUVA_{254}$	Chl a p=-0.424* (N=32) DOC p=-0.649** (N=28) POM p=-0.636** (N=32) Sal p=-0.381* (N=32) a_{350} p=0.410* (N=32)	Chl a p=-0.381* (N=32) DOC p=-0.803** (N=28) POM p=-0.725** (N=32) Sal p=-0.902** (N=32) Temp p=-0.704** (N=32) a_{350} p=0.908** (N=32) E_2:E_3 p=-0.566** (N=32) $S_{275-295}$ p=-0.650** (N=32) S_R p=-0.813** (N=32)
a_{350}	a_{254} p=0.982** (N=32)	POM p=-0.852** (N=32) DOC p=-0.638** (N=28) nitra+nitri p=0.394* (N=32) Sal p=-0.962** (N=32) Temp p=-0.853** (N=32) $SUVA_{254}$ p=0.908** (N=28) E_2:E_3 p=-0.772** (N=32 $S_{275-295}$ p=-0.826** (N=32) S_R p=-0.730* (N=32)
E2:E3	$S_{275-295}$ p=0.717** (N=32)	Chl a p=0.732** (N=32) POM p=0.759** (N=32) nitra+nitri p=-0.400** (N=32) Sal p=0.748** (N=32) Temp p=0.880** (N=32) $SUVA_{254}$ p=-0.566** (N=28) a_{350} p=-0.772** (N=32) $S_{275-295}$ p=0.950** (N=32) S_R p=0.368* (N=32)
S275-295	E_2:E_3 p=0.717** (N=32) S_R p=0.602* (N=32)	Chl a p=0.672** (N=32) POM p=0.738** (N=32) DOC p=0.424* (N=28) Nitra+nitri p=-0.392* (N=32) Sal p=0.801** (N=32) Temp p=0.870** (N=32) $SUVA_{254}$ p=-0.650** (N=28) a_{350} p=-0.826** (N=32) E_2:E_3 p=0.950** (N=32) S_R p=0.485** (N=32)
S_R	$S_{275-295}$ p=0.602* (N=32)	POM p=0.582** (N=32) DOC p=0.586** (N=28) Sal p=0.792** (N=32) Temp p=0.638** (N=32) $SUVA_{254}$ p=-0.813** (N=28) a_{350} p=-0.730** (N=32) E_2:E_3 p=0.368 $S_{275-295}$ p=0.485** (N=32)

**Correlation is significant at the 0.01 level (two-tailed); *Correlation is significant at the 0.05 level (two-tailed); Sal – salinity; Temp- temperature; Chl a- Chlorophyll a; SPM – suspended particulate matter; POM - particulate organic matter; DOC - dissolved organic carbon;

5.3.4. CORRELATIONS BETWEEN MICROBIAL PARAMETERS AND PHYSICOCHEMICAL AND SPECTRAL PROPERTIES

The values of the spearman correlation coefficient for microbial parameters and different physicochemical parameters and CDOM spectral characteristics are presented in Table 5-V. TBN at the station N1 was positively correlated with salinity, temperature and with the absorption coefficients at 254 and 350 nm. At the station I6, TBN was positively correlated with the concentration of DOC and negatively correlated with the concentration of nitrate plus nitrite and the slope $S_{350-400}$. BBP showed a negative correlation with nitrate plus nitrite, at bot stations. At the station N1, BBP correlated positively with temperature and the station I6with SPM concentration, the slope $S_{350-400}$ and the ration E_2:E_3. The activity of Leu-AMP correlated with a high number of CDOM spectral characteristics at both stations. Contrasting, the activity of ß-GlCase correlated positively with TBN and nitrate plus nitrite concentration and negatively with the ratio E_2:E_3 and the slope $S_{275-295}$ at station N1.

Table 5-V. Spearman correlations between total bacterial number (TBN), bacterial biomass production (BBP), aminopeptidase (Leu-AMPase) and ß-glucosidade (ß-GlCase) hydrolysis rates, and the different chemical and physical parameters at the marine and brackish water zones of the estuary Ria de Aveiro.

	Marine zone [N1]	Brackish water zone [I6]
TBN	Sal p=0.470** (N=32)	DOC p=0.516** (N=28)
	Temp p=0.420* (N=32)	NO_3^-+NO_2^- p=-0.361* (N=32)
	a_{254} p=0.425* (N=32)	
	a_{350} p=0.466** (N=32)	
	NO_3^-+NO_2^- p=-0.359* (N=32)	SPM p=0.372* (N=32)
BBP	Temp p=0.468** (N=32)	NO_3^-+NO_2^- p=-0.612** (N=32)
		E_2:E_3 p=0.350* (N=32)
Leu-AMPase	TBN p=0.673** (N=32)	Chl a p=0.559** (N=32)
	Chl a p=0.486** (N=32)	POM p=0.532** (N=32)
	Sal p=0.486** (N=32)	DOC p=0.672** (N=28)
	Temp p=0.536** (N=32)	Sal p=0.728** (N=32)
	a_{254} p=0.383** (N=32)	Temp p=0.743** (N=32)
	a_{350} p=0.379* (N=32)	$SUVA_{254}$ p=-0.893** (N=28)
	$S_{275-295}$ p=-0.441* (N=32)	a_{350} p=-0.686** (N=32)
		E_2:E_3 p=0.633** (N=32)
		$S_{275-295}$ p=0.654** (N=32)
		S_R p=0.791** (N=32)
ß-GlCase	TBN p=0.472* (N=28)	
	NO_3^-+NO_2^- p=0.438* (N=28)	
	E_2:E_3 p=-0.635** (N=28)	
	$S_{275-295}$ p=-0.662** (N=28)	

**Correlation is significant at the 0.01 level (two-tailed); *Correlation is significant at the 0.05 level (two-tailed); Sal – salinity; Temp - temperature; Chl a - Chlorophyll a; SPM – suspended particulate matter; POM - particulate organic matter; DOC - dissolved organic carbon;

5.3.5. MICROCOSM EXPERIMENTS

5.3.5.1. PHOTOCHEMICAL ALTERATIONS OF CDOM SPECTRAL CHARACTERISTICS

The variations of the different spectroscopic parameters of CDOM induced by natural sunlight irradiation in the long-term assays are presented in Table 5-VI. After 168h there was reduction in the absorption coefficients at 254 (a_{254}) and 350 (a_{350}) nm and increment of the values of the $S_{275-295}$, and of the ratios E_2:E_3 and S_R. Compared with the initial value, a_{254} in the natural sunlight treatment decreased 29 % and 9 % after 168 h in the assay 1 and 2, respectively. Contrastingly, in the dark control, the value of a_{254} increased 11 and 75 % after 168 h in the assay 1 and 2, respectively. The value of a_{350} nm showed similar pattern of variation, with higher decreases (68% - assay 1 and 60 % - assay 2) in the natural sunlight treatment and increases in the dark control (18% - assay 1 and 117 % - assay 2). The values of the spectral slope between 275 and 295 nm ($S_{275-295}$) of sunlight exposed-CDOM increased 75 % and 100 % in the assay 1 and 2, respectively, whereas in the dark control the values remain stable. The ratios E_2:E_3 and S_R increased considerably in the natural sunlight treatment. The exposure of CDOM to natural sunlight resulted in the increment of 140 and 125 % of the ratio E_2:E_3 in the assay 1 and 2, respectively. In the assays 1 and 2, the S_R ratio also increased 104 and 130 % in the natural sunlight treatment, respectively. In the dark control, the ratios showed E_2:E_3 and S_R slightly variations during the assays.

Table 5-VI. Variation of DOM spectroscopic characteristics of surface water samples irradiated with natural sunlight compared with dark controls. Water samples were collected at the marine zone (N1) of the estuary Ria de Aveiro in two different dates, May 2006 (assay 1) and June 2006 (assay 2).

Spectroscopic parameters	Irradiation time (h)	0		12		168	
	Assay	1	2	1	2	1	2
a_{254}	Sunlight	4.979	3.312	4.371	3.861	3.532	3.013
	Dark control			6.591	4.933	5.532	5.797
a_{350}	Sunlight	1.169	0.658	0.713	0.542	0.373	0.265
	Dark control			1.596	1.249	1.377	1.429
E_2:E_3	Sunlight	5.84	6.54	8.09	9.78	14.01	14.74
	Dark control			5.24	4.99	5.22	5.08
$S_{275-295}$	Sunlight	0.008	0.008	0.010	0.012	0.014	0.016
	Dark control			0.007	0.008	0.007	0.008
S_R	Sunlight	1.21	1.40	1.67	1.80	2.47	3.22
	Dark control			1.39	1.54	1.30	1.48

Photochemical alterations of the different spectroscopic parameters of CDOM induced by natural sunlight exposure during the short-term assay are shown in Table 5-VII. The exposure of DOM to natural sunlight during a short-term period (12 h) resulted in the increase of a_{254} and a_{350}

values. Compared with the initial value, a_{254} increased approximately 90 % in both natural sunlight and dark control treatments. a_{350} nm showed similar pattern of variation, increasing 66±2.7 % in the natural sunlight treatment and 107±2.5 % in the dark control. The values of the spectral slope between 275 and 295 nm ($S_{275-295}$) of sunlight exposed-CDOM increased 14 % in the natural sunlight treatment and 1.4 % in the dark control compared with the initial values. The ratios E_2:E_3 and S_R also showed slightly increases in the natural sunlight treatment, 13.5 and 17.6 % respectively.

Table 5-VII. Variation of DOM spectroscopic characteristics of surface water samples rooftop irradiated with natural sunlight compared with dark controls during a short period of exposure (12 h). Water sub-samples were collected at the marine zone (N1) of the estuary Ria de Aveiro in March 2009.

Irradiation time			0 h			12 h	
Spectroscopic parameters	Subsample	1	2	3	1	2	3
a_{254}	Sunlight	2.444	2.380	2.381	4.514	4.536	4.500
	Dark control		2.380	2.381	4.819	4.820	4.808
a_{350}	Sunlight	0.479	0.493	0.488	0.812	0.814	0.803
	Dark control		0.493	0.488	1.005	1.013	1.003
E_2:E_3	Sunlight	6.67	6.65	6.72	7.55	7.60	7.59
	Dark control		6.65	6.72	6.58	6.51	6.53
$S_{275-295}$	Sunlight	0.0072	0.0072	0.0072	0.0082	0.0082	0.0082
	Dark control		0.0072	0.0072	0.0073	0.0073	0.0073
S_R	Sunlight	0.85	0.85	0.86	1.00	1.00	1.01
	Dark control		0.85	0.86	0.89	0.89	0.89

5.3.5.2. RESPONSE OF BACTERIA TO IRRADIATED CDOM

Long-term assay: The incubation of bacteria with DOM irradiated during different times resulted in considerable higher values of biomass production values in the natural sunlight treatments compared with dark controls, in both assays and, in both 72 and 168 h of irradiation (Figure 5.2). Bacteria inoculated with non-irradiated DOM (0 h) increased their biomass production along of incubation time from 0.07 to 5.43 µg C L^{-1} h^{-1} in assay 1, and from 0.01 to 18.57 µg C L^{-1} h^{-1} in assay 2. Bacteria inoculated with 12 h-irradiated DOM increased their biomass production from 0.43 to 19.15 µg C L^{-1} h^{-1} in sunlight treatment and, from 0.43 to 9.55 µg C L^{-1} h^{-1} in the dark control in the assay 1. In the assay 2, bacterial biomass production increased from 0.45 to 22.41 µg C L^{-1} h^{-1} in the natural sunlight treatment and from 0.49 to 7.97 µg C L^{-1} h^{-1} in the dark control. Along of the incubation time, the response of bacteria to 168 h-irradiated DOM was higher in the natural sunlight treatment than in the dark control, in both assays. However, in assay 2 the response was faster compared than in assay 1, and the highest value (27.68 µg C L^{-1} h^{-1}) was observed in the natural sunlight treatment, at the 72 h.

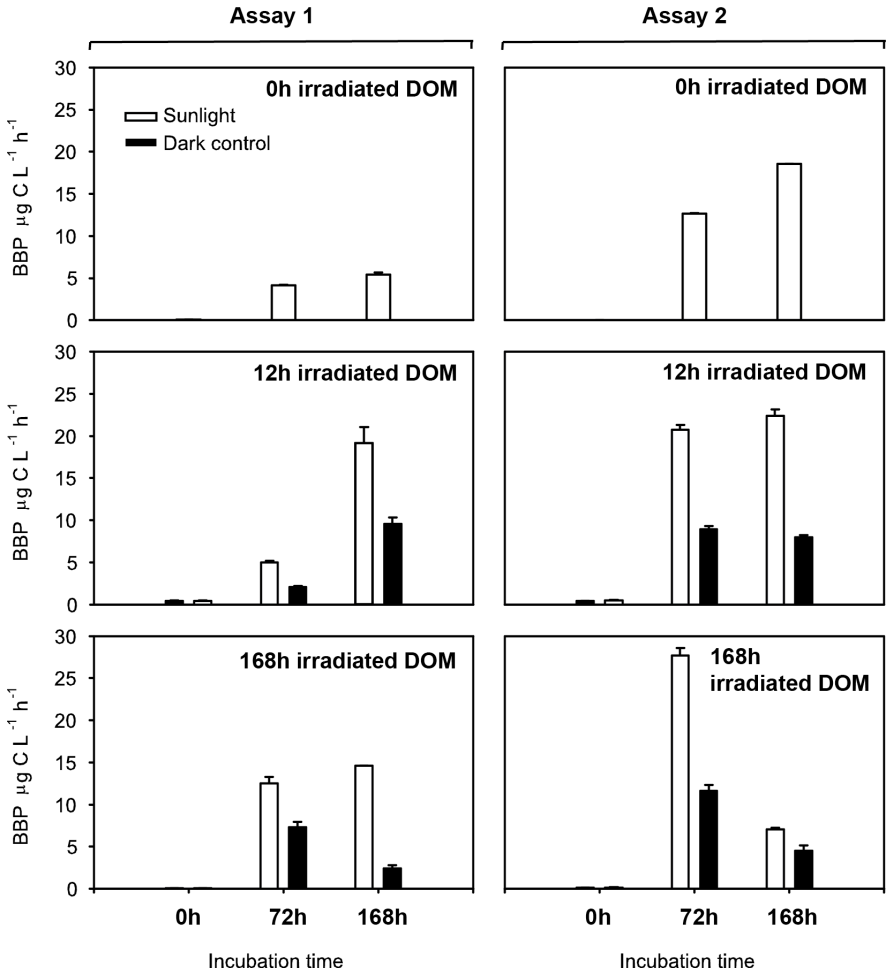

Figure 5.2. Variation of bacterial biomass production (BBP) along of the incubation assays with DOM exposed to different times of natural sunlight (long-term assays). Bars and respective errors represent the average and standard deviation of the 3 replicates, respectively.

Short-term assay: With the exception of Leu-AMPase, for which Hm values were higher in the natural sunlight treatment (range 324 - 2015 nmol L^{-1} h^{-1}) than in dark controls (range 357 - 1094 nmol L^{-1} h^{-1}), bacterial abundance, biomass production and ß-GlCase activity did not show a different response to sunlight irradiated DOM or to dark treatment (Figure 5-3). TBN (range 1.5 – 11.4 x10^8 cells L^{-1}) and BBP (range 0.36 – 23.3 µg C L^{-1} h^{-1}) showed a similar pattern, increasing in the first 72 h and decreasing after. The activity of ß-GlCase (range 12.2 - 76.0 nmol L^{-1} h^{-1}) showed a decreasing trend along of the incubation time, with both irradiated and non-irradiated DOM.

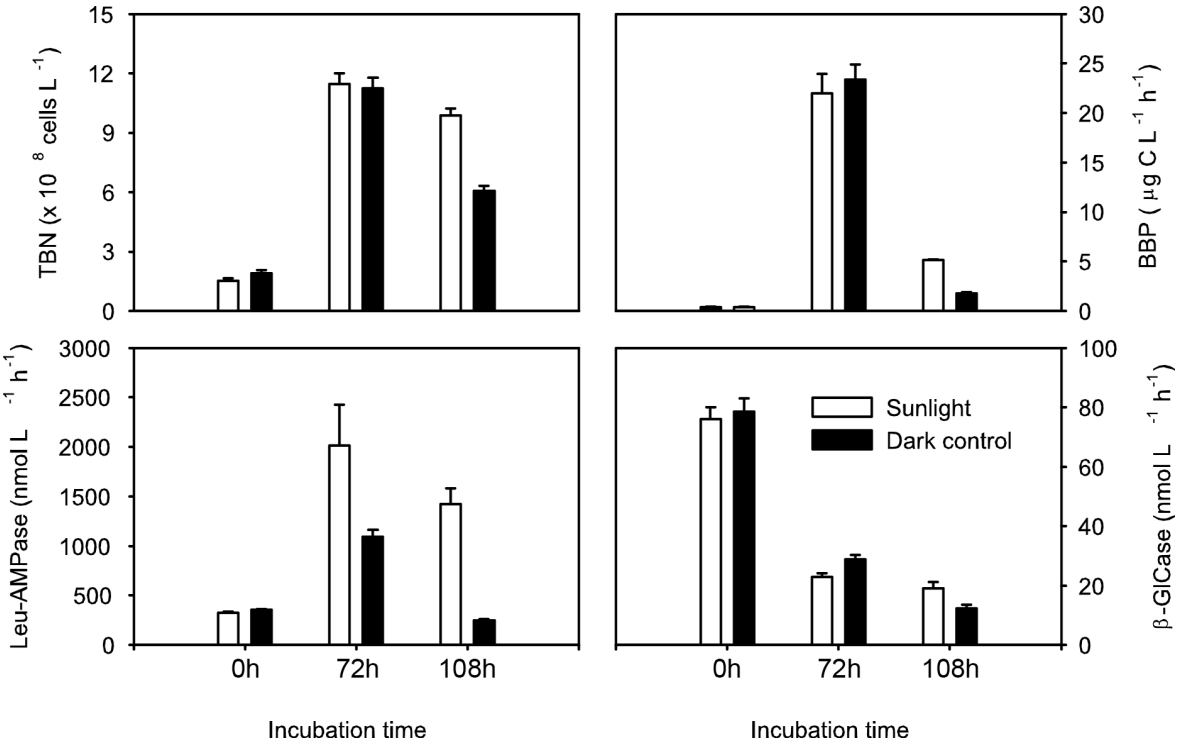

Figure 5.3. Variation of total bacterial number (TBN), bacterial biomass production (BBP) and, Hm of the enzymes aminopetidase (Leu-AMPase) and ß-glucosidase (ß-GlCase) along of the incubation assay with DOM exposed to 12 h of natural sunlight (short-term assay). Bars and respective errors represent the average and standard deviation of the 3 sub-samples, respectively.

5.4. DISCUSSION

5.4.1. CDOM OPTICAL PROPERTIES AT THE TWO ESTUARINE SITES

The CDOM of the marine and brackish water zones showed different spectral characteristics, reflecting the different amounts and prevailing sources of organic matter, as well as distinct riverine and oceanic influences. The CDOM at the brackish water zone showed significantly higher values of the $SUVA_{254}$ and a_{350} and lower ratio of SR compared with the marine zone, indicating that, at in this estuarine zone, DOM is composed by higher proportion of land derived materials (Hernes & Benner, 2003), with higher molecular weight (MW) (Helms *et al.*, 2008) and higher aromatic content (Weishaar *et al.*, 2003). The absorption coefficient at 350 nm has been used to trace land originated DOM inputs in coastal (Hernes & Benner, 2003) and estuarine systems (Spencer *et al.*, 2007b) due to its strong correlation with the dissolved lignin content (Hernes & Benner, 2003). In the present study, a strong negative correlation of both $SUVA_{254}$ and a_{350} parameters with salinity was observed, suggesting a riverine transport of this land-derived CDOM. This estuarine site is influenced by River Boco discharges at the end of the Ílhavo channel and, notwithstanding of its relative low contribution to the global freshwater inputs in Ria de Aveiro, its importance increase after periods of heavy precipitation (Dias *et al.*, 2003),

reducing water residence time (as shown in chapter 2). A seasonal pattern of variation of these spectroscopic parameters was highlighted by the correction with temperature, and their negative relation indicated relative higher amounts of allochthonous sources in DOM pool during the cold season, in comparison to the warm season.

The higher values of the ratios S_R and E_2:E_3 at the marine zone indicated that the DOM pool consists of lower MW compounds than in the brackish water zone. S_R and E_2:E_3 showed an increasing trend along of an estuarine salinity-increasing gradient, indicating a decrease in color as well as in the average the MW of DOM pool (Peuravuori & Pihlaja, 1997; Helms *et al.*, 2008; Stephens & Minor, 2010). However, a lack of correlation with abiotic factors and/or primary producers suggested that other factors contribute to the proportion of LMW compounds in the DOM pool, at this estuarine area. Contrastingly, at the brackish water zone, these ratios correlated positively with chlorophyll, POM and DOC concentrations, as well as with salinity and temperature. The typical increase of phytoplankton biomass at this estuarine section during the warm season, might contribute to increase the relative proportion of autochthonous DOC and POM, containing a higher proportion of LMW compounds (Zhang *et al.*, 2013).

5.4.2. Relation between bacterial abundance, activity and CDOM properties

The abundance of bacteria and Leu-AMPase activity in the water column of the marine zone showed positive correlations with the parameters a_{254} and a_{350}. The increase of abundance and proteolytic activity of bacterial communities with salinity and temperature during the low riverine influence (warm season), suggest the other relation than land-derived DOM inputs with absorbance of CDOM at 254 and 350 nm.

The increase of UV absorbance and of the humification index values of DOM after incubation with bacteria, were found to be more pronounced for algal- and plant-derived DOM compared with other sources (Hur *et al.*, 2009). Moreover, algal-derived DOM causes a substantial increase in the average MW value, whereas little changes or even decreases in the MW values were observed for terrestrial sources of DOM (Hur, 2011). Guillemette and del Giorgio (2012) also observed a production of humic-like fractions during incubations of bacteria with DOM, with rates vary in function of bacterial growth efficiency and concentration of inorganic nutrients. At this estuarine area, bacterial abundance and proteolytic activity have a typical seasonal pattern of variation, related with a strong increase of primary production during the warm season (Cunha *et al.*, 2000; Almeida *et al.*, 2002a). A selective consumption of LMW algal-derived and a simultaneous production of HMW DOM might increase the UV absorbance of CDOM, explaining the relation with a_{254} and a_{350} parameters.

At the brackish water zone, bacteria abundance was significantly related with the concentrations of DOC and inorganic nitrogen, and Leu-AMPase activity correlated with phytoplankton biomass and both particulate and dissolved organic concentrations. At this estuarine area, Leu-AMPase activity also showed a positive correlation with E_2:E_3 and S_R ratios, supporting a phytoplankton production of proteic-rich DOM (Myklestad, 2000), with LMW and aromaticity. At the marine zone, ß-GlCase correlated negatively with S_R and E_2:E_3 ratios, suggesting a stimulation by HMW DOM, probably supplied by freshwater inputs, indicated by the positive correlation with inorganic nitrogen.

5.4.3. BACTERIAL RESPONSES TO PHOTOCHEMICAL CHANGES IN CDOM

In aquatic systems, the absorption of light by CDOM results in significant changes on its optical properties (Helms *et al.*, 2008; Zhang *et al.*, 2009). In the present study we observed pronounced changes on CDOM optical properties during the long-term assays but only slight variations in the short-term assay. The first explanation would be the different times of DOM sunlight-irradiation, but the selected spectral parameters showed considerable changes after 12 h in the long-term assays, the same time of sunlight irradiation in the short-term assay. Therefore, other factors, rather than light dose, may underlie the distinct photoreactivity of CDOM. The sunlight-irradiation of DOM can reduce or increase the bioavailability of the exposed DOM, depending of the initial content of labile or refractory DOM (Obernosterer *et al.*, 1999; Tedetti *et al.*, 2009). The comparison of the initial values of the selected spectral parameters in the short and long-term assays showed that water samples used in the long-term assays setup had higher aromatic content (a_{254}, a_{350}) and MW (lower E_2:E_3) suggesting a more refractory nature, and therefore susceptible of higher photochemical alterations (Benner & Ziegler, 1999). A different seasonal photochemical reactivity of CDOM was observed previously at this estuarine system (Pinto et al, unpublished data).

The decrease of the values of the absorption coefficients at 254 (a_{254}) and 350 (a_{350}) nm and in the increase of the values of the $S_{275\text{-}295}$, and of the ratios E_2:E_3 and S_R indicate that CDOM suffered significant transformations during the irradiation with sunlight in the long-term assays. The slope ratio SR increased in the photochemical process, and therefore could be used as an indicator of photobleaching and composition change of CDOM (Helms *et al.*, 2008; Zhang *et al.*, 2009). A photochemical degradation and decrease of DOM MW was also indicated by the increase of ratio E_2:E_3 during sunlight exposure. Changes in MW are inversely correlated with the ratio of the absorbance at 250 nm to that at 365 nm (E_2:E_3) (Lou & Xie, 2006). The exposure of DOM to sunlight increased its bioavailability, stimulating bacterial activity, probably due to reduction of MW. Bacterial biomass production was twice as high at light treatment, compared with dark

control in the long-term assays.

In the short-term assay, the small changes of CDOM properties only promote significant different responses in sunlight treatment when compared with dark controls in the activity of Leu-AMPase. Smith and Benner (2005) observed that bacterial carbon metabolism of photo-altered DOM is coupled to an enhanced demand for inorganic nutrients, which may induce an N-limitation in sunlight treatment, and consequent stimulation of proteolytic activity. A positive correlation between aminopeptidase activity and N limitation has been observed in this estuary and inferred as an indication of organic N-sources utilization by bacteria (Cunha & Almeida, 2009). In this study, the concentration of inorganic nutrients was not determined, and therefore we can only speculate about the possible occurrence of N-limitation during the experiments. Furthers investigations are needed to clarify a relation between nutrients and responses of estuarine bacteria to photochemical transformations.

5.5. CONCLUSION

In estuarine system Ria de Aveiro, both photochemical and microbial processes yielded optical changes in CDOM. The magnitude of photochemical and microbial induced CDOM changes is dependent on the initial characteristics of DOM pool. A more “labile” DOM pool might undergo lower photochemical alterations and stimulate the production of refractory compounds by bacteria, whereas, a more “refractory” DOM pool might experience substantial photochemical changes, increasing its bioavailability and recycling. Considering the seasonal and hydrological influences in the relative proportions of the “labile” and “refractory” fractions in the DOM pool, the importance of these processes might vary as well annually and inter-annually. The overall result of these combined photochemical and microbial effects determine the bioavailability and fate of CDOM in the estuarine system and have influence on local productivity and in adjacent coastal areas. A further investigation, incorporating the experimental simulation of photochemical and microbial processes in the shallow and turbid areas of the estuary will allow obtain a more comprehensive knowledge of these processes in this estuary and in other similar aquatic ecosystems.

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CHAPTER 6

6. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVE

In the estuarine system Ria de Aveiro, the abundance and heterotrophic activity of bacteria are influenced by physical factors, namely those related with hydrology and light exposure. Hydrological features, such as freshwater inputs and water currents intensity determine longitudinal and vertical patterns of distribution and exert direct and indirect influence on the activity of estuarine bacteria. Hydrodynamics also determines the amount and prevailing nature of organic matter, as well as the concentrations of inorganic compounds, which in turn influence the rate and mechanism of photochemical and microbial processes. The different quantity and quality of organic matter in combination with local hydrological characteristics determine the rates of polymer hydrolysis and monomer incorporation and the prevailing patterns of heterotrophic activity of bacterioneuston and bacterioplankton.

Freshwater inputs to the estuary are a major factor of direct and indirect regulation of bacterial dynamics. The pattern of variation of total bacterial abundance and activity in relation to freshwater inputs was different at the marine and brackish water zones. At the marine zone, the total bacterial number increased as the influence of freshwater decreased, but at the brackish water zone, bacterial abundance showed a concave parabolic pattern of variation, with high abundances at most extreme hydrological regimes, and lower at the transitional situations between wet and dry seasons. Bacterial biomass production at the marine zone decreased from high to medium influence of freshwater, increasing to maximum values under low influence whereas, at the brackish water zone, the average values were similar with no clear trend related with the hydrological regime.

A phytoplankton enrichment at the bottom layer and bacterial enrichment at the top layer of the stratified water column, during high freshwater inputs at the marine zone, suggests opposite fluxes of phytoplankton and bacterioplankton cells, following the inflow of bottom salty water and the outflow of the top brackish water layer, respectively. At the brackish water zone, a negative correlation with salinity and an advection time shorter than the doubling time of phytoplankton biomass supports the hypothesis of freshwater source for phytoplankton.

High freshwater inputs increase are associated with the increase of particle-attached bacteria, and its dynamics is highly impacted by advective transport induced by freshwater inflow, and resuspension processes. The intensity of these physical processes might determine the degree of differentiation between the structure of free and particles-attached bacterial communities.

The activity of estuarine bacteria is controlled by different nitrogen concentrations, resulting from different freshwater inputs, which, in association with different prevailing sources of organic substrates, induces significant changes in bacterial biomass production. High concentrations of DIN supplied by the high inputs of freshwater, inhibit bacterial biomass production, perhaps by inhibition of amino acid incorporation. Low freshwater inputs and low DIN concentrations stimulated a bacterial activity requirement of DIN uptake, fomented by a high concentration of nitrogen-poor organic compounds.

The hydrodynamic characteristics of the two estuarine sites also influenced the distribution and heterotrophic activities in the SML. At the marine zone, the vertical mixing forced by the hydrodynamic conditions, inhibited the incorporation of monomers, bacterial biomass production and promoted the interchange of bacterial cells between the SML and the UW column. The stronger hydrodynamics of this estuarine zone, allied to a less productive water column, also promoted intense hydrolytic activities at the SML environment. Consistent and significantly higher hydrolysis rates of the enzymes ß-glucosidase, ß-galactosidase, alkaline phosphatase and lipase were observed in the SML at the marine zone of the estuary. At the brackish water zone, the hydrodynamic stability reduces vertical mixing, increasing the residence time of the bacterial community at surface, promoting the enrichment of the SML in nutrients and explaining the higher rates of bacterial biomass productivity in comparison to UW. The hydrodynamic stability and a more productive water column lead to similar bacterioneuston and bacterioplankton heterotrophic activities. The degree of differentiation between heterotrophic activities of bacterial communities in the SML and UW in estuarine systems is, therefore, a result of the different organic enrichments at the air-water interface and the intensity of exposure to physical processes, among other factors.

The estuarine SML environment favors the ‘attached’ way of life. The fraction of particle-attached bacteria was significantly higher at the SML, both in the marine and in the brackish water zones of the estuary. On average, particle-attached bacteria represent 20% and 40% of the total abundance at the SML, in marine and brackish water zones, respectively. The dynamics of particle-attached bacteria at SML and UW was influenced by wind. In both zones, the number of particle-attached bacteria in the water column increased with increasing wind intensity.

The CDOM of the marine and brackish water zones showed different nature as inferred from the spectral characteristics, reflecting the different availability and prevailing sources of organic matter, as well as distinct riverine and oceanic influences. The CDOM at the brackish water zone showed significantly higher values of the $SUVA_{254}$ and a_{350} and lower ratio of SR compared with the marine zone, indicating that, at in this estuarine zone, DOM is composed by higher proportion of land derived materials, with higher molecular weight and higher aromatic content.

Photochemical and microbial processes yielded optical changes in CDOM in the estuary. In

the present study we observed pronounced changes on CDOM optical properties resulting from the irradiation of DOM with natural sunlight. The magnitude of photochemical effects was dependent on the initial characteristics of DOM pool. A more "labile" DOM pool might undergo lower photochemical alterations, whereas, a more "refractory" DOM pool might experience substantial photochemical changes. The exposure of DOM to sunlight increased its bioavailability, stimulating bacterial activity, probably due to an overall reduction of the MW. Bacterial biomass production was twice as high in light-treated water, compared with dark control. Microbial induced changes in CDOM were also dependent on the initial characteristics of DOM pool. A "labile" DOM pool can stimulate the production of refractory compounds by bacteria, which combined with a selective consumption of LMW algal-derived organic matter increases the UV absorbance of CDOM. Considering the seasonal and hydrological influences in the relative proportions of the "labile" and "refractory" fractions in the DOM pool, the importance of these processes might vary annually and inter-annually. The overall result of combined photochemical and microbial effects determine the bioavailability and fate of CDOM in the estuarine system and have influence on local productivity and in adjacent coastal areas.

The present work consolidated the knowledge about the impact of freshwater inputs on estuarine bacterial communities, allowing the anticipation of the possible outcome of changes in freshwater inputs related with climate changes or/and regional freshwater management, in terms of estuarine biogeochemical cycles. This study also showed that estuarine dynamics significantly influences microbial activity at the air–water interface, explaining differences in bacterial activity and density between different sites within a same system and, possibly, between different systems. Both photochemical and microbial processes yielded changes in optical properties of CDOM and the overall result of these combined effects determines the bioavailability and fate of CDOM in the estuarine system and has influence on local productivity and in adjacent coastal areas.

The present study also raised some relevant questions that need to be addressed in future. One of the uncertainties that persist is about the impact resuspension processes and advective transport induced by freshwater inflow in the degree of differentiation between free and particle-attached bacterial communities in the estuary. A comprehensive study addressing the physical influence on diversity of these two estuarine communities by high-throughput pyrosequencing analysis, will most certainly provide valuable answers.

The factors (number or/and nature of the particles) that promote the attachment of bacteria to particles in the SML environment, as well as the mechanisms by which hydrodynamic and meteorological events promote mixing in the water column, influencing the dynamic and activity of bacterial communities, remain to be elucidated. In order to answer these relevant questions, laboratory experiments will be conducted and individual and combined effects will be simulated.

Fluxes of bacteria and phytoplankton between the estuary and ocean have been estimated before during a period with a salty homogeneous water column. However, a re-estimation of those fluxes in defined periods of stratification of the water column by the application of a 3-D numerical model, would allow a more accurate estimation of the impact of estuarine system on the adjacent coastal waters.

The integration of the experimental results of the simulation of photochemical and microbial processes in the shallow and turbid areas of the estuary will provide a more comprehensive understanding of these processes in Ria de Aveiro and in other similar aquatic ecosystems. Simulations under controlled conditions of temperature and radiation intensity with "solar box" equipment are a convenient approach for conducting reproducible experiments and addressing several scientific questions that remain to be investigate, such as the impact of photo-alterations of DOM on microbial diversity in the estuary, the importance of reactive oxygen species (ROS) in DOM photoreactions and their impact on bacterial activity. The actual impact of photochemical process in the estuary will be the net balance between growth promoting and growth inhibiting factors (ROS).

The mechanisms involved in the control of activity of estuarine bacteria by nitrogen availability and their association with different sources of organic substrates remains to be clarified. Furthermore, a possible enhanced bacterial demand for inorganic nutrients promoted by the photo-altered DOM can also be possible. Microcosm experiments, in which bacteria are inoculated on different sources of DOM, including photo-altered DOM, under different concentrations of inorganic nutrients will allow to elucidate those mechanisms.

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